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THE PARTIAL PURIFICATION OF THE ESTERASE IN PIG'S LIVER.

BY GEORGE PEIRCE.

(From the Physiological Laboratory of the University of Wisconsin.)

(Received for publication, August 1, 1913.)

The preparation of a substance which can be regarded as an enzyme free from foreign material has never been accomplished. Invertases and proteases of considerable strength have been prepared, but very little work has been done on the purification of lipases. The preliminary report of work with the esterase from pig's liver may therefore be of interest.

The crude enzyme solution was prepared in much the same manner as in previous investigations. One hundred grams of fresh pig's liver were ground with sand and water, strained through cloth and made up to 1 liter with distilled water. Toluene was added as a preservative. After incubation at 37° for one day and after several weeks' standing at room temperature it was filtered through a folded filter until clear. The filtrate will be referred to as 10 per cent crude enzyme solution. 20 per cent solutions were also made.

The crude enzyme solution was dialyzed in collodion bags for five or six days and filtered. About 90 per cent of the solid substance was removed by this process, and the solution lost about 20 per cent of its total activity so that the purification was considerable. This solution will be referred to as dialyzed enzyme solution.

In it was now dissolved one-half the amount of ammonium sulphate necessary for complete saturation, and the solution was poured repeatedly through the same folded filter until a clear filtrate was obtained. The precipitate was practically inactive and was rejected. The filtrate was then fully saturated with ammonium sulphate and filtered till clear. The filtrate was inactive. The precipitate was taken up in water and the solution dialyzed till it no longer gave a turbidity with BaCl_2 . This

represents the most highly purified solution obtained (Solution B). No attempt was made to get a solid active substance.

A small portion of one of these solutions was mixed with an ethyl butyrate solution about two-thirds saturated. Both solutions had been previously warmed to 37° and the reaction was carried on at this temperature. The time of the beginning of the reaction (which was not more than three seconds in error) was taken at the time when one-half of the enzyme had flowed from a pipette into the ethyl butyrate solution. At suitable intervals 50 cc. were removed with a pipette and allowed to flow into an Erlenmeyer flask containing 5 cc. of 1 per cent neutralized NaF. The mixture was titrated with $\frac{N}{20}$ NaOH free from carbonate to a moderately deep shade of pink, phenolphthalein being used as the indicator. NaF of the above strength inhibits the enzyme completely in acid solutions, but allows the hydrolysis to proceed slowly in neutral or faintly alkaline solutions. Consequently the titrations need not be made immediately, but the final stage must be performed in a few seconds. This was one of the reasons for the selection of a deeper shade of pink than usual for an end point. The time of completion of the reaction is taken when the first drop from the pipette flows into the NaF. This allows for the slowing of the hydrolysis by cooling during the pipetting and for progress of the reaction in that part of the reaction mixture that has not yet been mixed with the NaF. The error in the measurement of the time was probably less than ten seconds in all. The titration error probably averaged about 0.1 cc. or a little less. Proper allowance was always made for any initial acidity. The temperature regulation was efficient and no error is to be attributed to this source.

The activities of the enzyme solutions are represented on the basis of their solid content. It has been previously shown¹ that in solutions of equal acidity, a given amount of enzyme hydrolyzes ethyl butyrate with the same absolute velocity over a wide range of enzyme and ester concentration. Consequently it will be convenient for the present purpose to compare the activities of the different solutions by giving the number of parts of ethyl butyrate that one part of solid substance hydrolyzes per hour. The ethyl butyrate concentration must always be above $\frac{2}{300}$ and

¹ Peirce *Journ. Amer. Chem. Soc.*, xxxii, pp. 1525 and 1530, 1910.

the acidity must be the same in the solutions to be compared. In this paper it will be understood that the initial acidity is "0" and the final acidity "10," *i.e.*, 10 cc. of $\frac{N}{20}$ acid per 50 cc. of reaction mixture.

15 cc. of Solution B contained 4.8 mgms. solid substance (dried over water bath).

20 cc. of this solution in a total volume of 560 cc. hydrolyzed 650 mgms. ethyl butyrate in 28.1 minutes (final acidity "10").

The activities of the various solutions follow:

DESIGNATION OF ENZYME SOLUTION	PARTS OF ETHYL BUTYRATE HYDROLYZED PER HOUR BY 1 PART SOLID SUBSTANCE
A ₁ . Crude 10 per cent.	10*
A ₂ . Dialyzed 10 per cent.	90
A ₃ . Partially† purified 10 per cent.	165
B. Purified 20 per cent.	217

* Initial acidity 1.4, final acidity 11.4.

† The half saturation with ammonium sulphate was omitted.

Kastle, Johnston and Elvove² prepared a clear esterase solution and estimated its activity on the basis of its solid content. Without any allowance for the different conditions of the hydrolysis, the solid substance in solution B was 400 times as active as that in their solution. Making a liberal allowance for differences in the conditions it seems safe to say that the solid substance of solution B was 50 and probably 100 times as active as theirs.

No detailed chemical investigation was made of the dried material from these enzyme solutions, owing to the small amount of material available. A₃ contained about 0.6 per cent phosphorus, but the figure is only approximate. An accurate determination of the tyrosine content of B was made. 4.8 mgms. of the dried substance were heated on a water bath for several hours with 20 per cent HCl, the HCl was evaporated and the residue taken up with a few drops of dilute HCl. A determination according to Folin, using one-quarter of the given quantity of reagents and diluting to 25 cc., gave the following result:

4.8 mgms. dried substance gave 0.283 mgm. tyrosine. Tyrosine=5.9 per cent.

² *Amer. Chem. Journ.*, xxxi, p. 526, 1904.

THE COMPOUND FORMED BETWEEN ESTERASE AND SODIUM FLUORIDE.

BY GEORGE PEIRCE.

(From the Physiological Laboratory of the University of Wisconsin.)

(Received for publication, August 1, 1913.)

In previous papers by Kastle and Loevenhart¹ and Loevenhart and Peirce² it was shown that sodium fluoride has a remarkable inhibiting action on lipases and esterases. In the present paper it is proposed to show: (1) that this inhibition is due to the formation of an inactive compound composed wholly, or in part, of sodium fluoride and the enzyme; (2) that the reaction in which this compound is formed, is reversible; (3) that an equation for the reaction based on the mass law, agrees with the observations.

The particular case investigated was that of the action of the esterase from pig's liver on ethyl butyrate. The choice was made on account of ease of experimentation and it is planned later to extend the work to other simple esters, and, if possible, to the true fats. Four different enzyme solutions were used. For the methods of their preparation the previous paper in this number of the *Journal* must be consulted. Preparation A was the crude 10 per cent enzyme there referred to, while B was the most highly purified enzyme obtained in that investigation. The crude 10 per cent enzyme solution used in Table III was about one-third as active as A. C was prepared in the same manner as B but was not quite so active.

The technique was the same as that of the previous paper except in Table V, where alcohol instead of sodium fluoride was used to stop the reaction before titration. When sodium fluoride was used in a reaction mixture it was mixed with the ethyl butyrate before the enzyme was added.

¹ *Amer. Chem. Journ.*, xxiv, p. 491, 1900.

² *This Journal*, ii, p. 397, 1907.

It has been shown³ that the concentration of ethyl butyrate makes practically no difference in the absolute velocity of acid production when no sodium fluoride is present. This has not been shown to be true in sodium fluoride mixtures, so to obviate any difficulty the initial concentration of ethyl butyrate is the same in any given set of experiments. It varied from $\frac{N}{25}$ to $\frac{N}{30}$ in different sets of experiments.

Using enzyme solution B several series were run, alike in all respects except that the concentration of the sodium fluoride varied. The numerical data for this series of experiments are given in Table I, and the results shown graphically in Figure 1. x is the

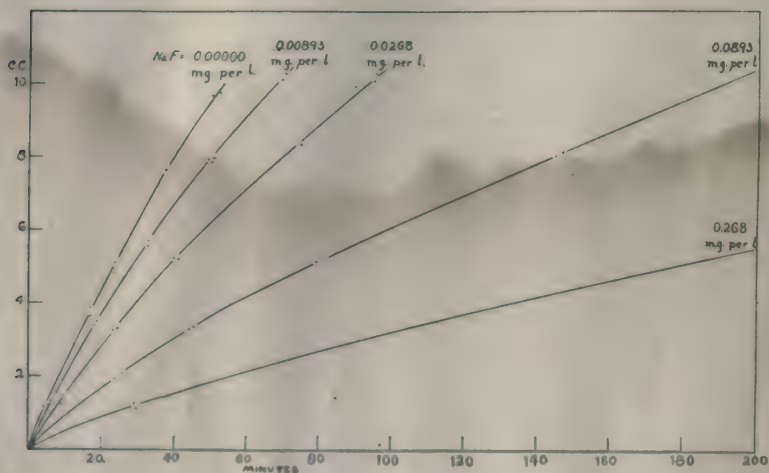


FIG. 1. TO ILLUSTRATE TABLE I.

number of cc. of $\frac{N}{20}$ butyric acid produced in 50 cc. of reaction mixture in t minutes. If the decimal point is moved two places to the left, the normality of the solution is obtained. The time taken to produce 10 cc. of acid is calculated for each series and reappears in the third column of Table II. The reaction mixtures were made up as follows:

- 250 cc. ethyl butyrate solution.
- 5 cc. enzyme solution B.
- 25 cc. { sodium fluoride solution of different strengths.
water.

³ Peirce: *Journ. Amer. Chem. Soc.*, xxxii, p. 1525, 1910.

TABLE I.

NaF = 0.00000 MGM. PER LITER				NaF = 0.00893 MGM. PER LITER			
Observed		Average		Observed		Average	
<i>x</i>	<i>t</i>	<i>x</i>	<i>t</i>	<i>x</i>	<i>t</i>	<i>x</i>	<i>t</i>
1.22	5.22	1.18	5.03	1.29	5.92	1.32	6.52
1.14	4.83			1.34	6.92		
3.62	16.92	3.73	17.02	3.48	18.17	3.40	18.00
3.84	17.12			3.31	17.83		
4.84	23.17	4.97	23.56	5.65	32.75	5.62	32.63
5.10	23.95			5.58	32.50		
7.62	37.42	7.64	37.52	7.92	49.30	7.96	49.69
7.65	37.62			7.99	50.08		
9.64	50.55	9.69	51.43			(10.00)	(67.8)
9.73	52.30			10.10	69.00		
		(10.00)	(53.7)		70.08	10.19	69.54
				10.27			
NaF = 0.0268 MGM. PER LITER				NaF = 0.0893 MGM. PER LITER			
Observed		Average		Observed		Average	
<i>x</i>	<i>t</i>	<i>x</i>	<i>t</i>	<i>x</i>	<i>t</i>	<i>x</i>	<i>t</i>
1.22	8.77	1.33	9.11	2.09	25.70	2.01	24.69
1.44	9.45			1.92	23.67		
3.21	22.97	3.26	23.55	3.34	44.92	3.30	44.46
3.31	24.12			3.26	44.00		
5.12	39.85	5.12	40.28	5.21	80.88	5.16	79.78
5.12	40.70			5.10	78.67		
8.33	74.47	8.33	74.38	8.15	146.78	8.08	145.56
8.32	74.28			8.01	144.33		
		(10.00)	(92.8)			(10.00)	(190.)
10.09	94.28	10.18	94.83	10.52	202.83	10.47	201.42
10.26	95.37			10.42	200.00		
NaF = 0.268 MGM. PER LITER							
Observed		Average					
<i>x</i>	<i>t</i>	<i>x</i>	<i>t</i>				
1.14	29.08	1.17	29.04				
1.20	29.00						
3.85	133.58	4.00	133.21				
4.15	132.83						
5.65	212.83	5.70	213.23				
5.75	213.62						
7.96	338.08	8.01	338.29				
8.06	338.50						
		(10.00)	(450.)				
10.39	479.42	10.51	479.77				
10.62	479.92						

The most obvious explanation for this inhibition is that a certain amount of the enzyme is inactivated by the sodium fluoride, either by destruction, or by the formation of some sort of inactive compound. We shall see later that the view that the enzyme is destroyed is untenable.

Any compound of sodium fluoride and esterase should be formed in accordance with the mass law:

$$(\text{Conc. free Enz.})^m \times (\text{conc. free NaF})^n = k (\text{conc. NaF. Enz.})^p \dots [1]$$

where m , n and p represent the number of molecules of the substances involved in the reaction.

Transposing:

$$k = \frac{(\text{conc. free Enz.})^m}{(\text{conc. NaF. Enz.})^p} \times (\text{conc. free NaF})^n \dots [2]$$

We have no data at present for assigning values to m , n and p , but a little consideration will enable us to exclude several possibilities at the outset and leave only a few others to be tested. It will be shown experimentally in Table V that the formation of the inactive compound is reversible. It is difficult to conceive of a reaction, wherein one molecule of active enzyme would be broken up by sodium fluoride into two or more molecules of an inactive compound, in such a manner that the reaction could be reversible. This would indicate that in equations [1] and [2] m is equal to or greater than p . For simplicity we can let $p = 1$ and $m = 1, 2, 3$ or more. The higher values are, of course, increasingly improbable. No values can be assigned to n on such considerations as these.

On the basis of the figures already obtained, the most simple equation possible will first be tested:

$$k = \frac{\text{conc. free Enz.}}{\text{conc. NaF. Enz.}} \times \text{conc. free NaF} \dots [3]$$

That is, we assume that one molecule of sodium fluoride combines with one molecule of enzyme to form one molecule of the inactive compound. The term $\frac{\text{conc. free Enz.}}{\text{conc. NaF. Enz.}}$ is calculated as follows:

It has been shown repeatedly⁴ that the absolute velocity of acid

⁴ Cf. Peirce: *Journ. Amer. Chem. Soc.*, xxxii, p. 1529, 1910.

production is very nearly proportional to the amount of enzyme present. In any event, a solution whose activity is inhibited by sodium fluoride behaves as if only a certain percentage of the total enzyme present were really acting. Let x represent this percentage. Then since the total percentage present is 100, the percentage of the NaF. Enz. present is $100 - x$ and $\frac{\text{conc. free Enz.}}{\text{conc. NaF. Enz.}} =$

$\frac{x}{100 - x}$. For example it took a solution containing no sodium fluoride 60 minutes to produce 10 cc. of $\frac{N}{20}$ acid, and a solution containing 1:40,000,000 sodium fluoride 100 minutes to produce the same amount. The one containing sodium fluoride had therefore $\frac{60}{100} = 60$ per cent of the activity of the other. If the activity as measured in this way is proportional to the active enzyme present, we may say that 60 per cent is "free" and 40 per cent combined with sodium fluoride. The ratio⁵ $\frac{\text{conc. free Enz.}}{\text{conc. NaF. Enz.}}$ is

⁵ The velocity falls off during the reaction so that the activity as measured by the reciprocal of the time taken to attain a given stage of the reaction represents the average activity during this period. Since the curves with different strengths of sodium fluoride are not exactly similar in shape a slight inaccuracy is involved. Absolutely accurate results can theoretically be obtained by plotting the curves with x and t as ordinate and abscissa respectively, and taking the values of the tangents $\frac{dx}{dt}$ at the same values of x on the different curves as proportional to the values of the activities at these points. Since now the curves have a nearly constant curvature, the value of $\frac{x}{t}$ for $x = 10$ will be very nearly equal to $\frac{dx}{dt}$ for $x = 5$. The simpler procedure is used for two reasons. First, the measurements are not sufficiently accurate to justify the labor involved in making an extremely accurate graphical measurement and, secondly, the method employed is absolutely objective.

Expressed as non-mathematically as possible the argument runs: We wish to obtain the ratio of the activities of two solutions, one containing NaF and the other containing none. The rate of hydrolysis diminishes as the reaction proceeds, but we can represent the average activity during the production of the first 10 cc. of acid by the reciprocal of the time taken to produce that acidity. *Considering the form of the curves*, this average activity will, with sufficient accuracy for our present purposes, be also the actual activity at the point where 5 cc. of acid has been developed.

therefore $\frac{60}{40} = 1.5$. The concentration of the *free* sodium fluoride cannot be obtained directly, but it will be taken as equal to the total sodium fluoride present. This is, of course, not absolutely true; but we shall see in Table IV that very little of the total sodium fluoride present is combined with the enzyme, especially in weak enzyme mixtures. No appreciable error is therefore involved.

The headings in the following table are all self-explanatory. The data are derived from Table I.

TABLE II.

5 cc. enzyme solution B in a total volume of 280 cc.

NaF		TIME TO REACH ACIDITY "10"	CONC. OF ENZYME PERCENTAGES		Free Ens. NaF. Enz.	$k \times 10^6$
Mgm. per liter	Normality		Free	NaF. Enz.		
minutes						
0.00000	0.000	53.7	100.0	0.0		
0.00893	0.213×10^{-6}	67.8	79.2	20.8	3.81	0.812
0.0268	0.638×10^{-6}	92.8	57.9	42.1	1.38	0.880
0.0893	2.13×10^{-6}	190.0	28.3	71.7	0.395	0.841
0.268	6.38×10^{-6}	450.0	11.94	88.06	0.136	0.868

* Acidity "10" = 10 cc. $\frac{N}{10}$ acid in 50 cc. mixture.

As an additional example a similar experiment with a crude 10 per cent extract is included. Only the final results are given. k is different in the two tables. This is due in part to slightly

TABLE III.

5 cc. enzyme solution used to 250 cc. ethyl butyrate solution.

NaF		TIME TO REACH ACIDITY "5"	CONC. OF ENZYME PERCENTAGES		Free Ens. NaF. Ens.	$k \times 10^6$
Mgm. per liter	Normality		Free Ens.	NaF. Ens.		
minutes						
0.0008	0.000	47.9	100	0		
0.0008	0.233×10^{-6}	53.9	89	11	8.09	1.88
0.0195	0.464×10^{-6}	58.5	82	18	4.56	2.12
0.0481	1.15×10^{-6}	77.1	62	38	1.64	1.87
0.0943	2.25×10^{-6}	105.4	46	54	0.852	1.92
0.189	4.50×10^{-6}	158.3	30	70	0.420	1.93

different conditions in the two experiments, but mainly to the fact that the enzyme solutions used were entirely different. Other experiments, performed with purified enzyme solutions, gave constants nearly equal to the ones in Table II. In every series, moreover, the values of k are constant within the limits of error of the experiment, so that the data agree satisfactorily with equation [3]. No other values for m , n and p in equations [1] and [2] are so consistent with the observations.

If the equations as given are true the ratio⁶ $\frac{\text{NaF. Enz.}}{\text{Free Enz.}}$ is proportional to the free sodium fluoride. If a large amount of enzyme is used we should expect so much of the sodium fluoride to be combined that the concentration of the free sodium fluoride would be appreciably diminished. In this event, a given concentration of sodium fluoride would have less inhibiting effect in strong enzyme solutions than in weak ones. On testing this view it was found that the inhibition was apparently less in very strong solutions, but the difference was so slight as to be within the limits of experimental error. Unfortunately no great quantity of uniform purified enzyme remained for experiments in duplicate, and as it was quite evident that the enzyme was far from pure, it did not seem advisable to repeat the experiment until a much purer enzyme could be obtained. The experiment did, however, show that very little sodium fluoride was bound even in enzyme solutions of considerable strength (five times the concentration in Table II), so that the assumption made in that experiment, that the free sodium fluoride was very nearly equal to the total sodium fluoride, is justified.

The following table gives the data on which the preceding conclusion is based. Only the last column requires any explanation. This is obtained as follows: The top figures of the first five columns are obtained by extrapolation, and thus a series of figures is obtained in the fifth column giving an irregularly descending series. The total amount of sodium fluoride present is 0.030 mg. per liter, and, starting from this figure, the last column gives a regularly descending series almost directly proportional to the figures in the next to the last column.

⁶ Note inversion of this ratio. This is done for convenience of presentation.

TABLE IV.

20 per cent purified enzyme B.
Total volume 280 cc.

0.714×10^{-6} N.
0.030 mgm. per liter.

CONCENTRATION OF ENZYME ABSOLUTE AMOUNTS		CONC. OF ENZYME PERCENTAGES		NaF. Ens. Free Ens.	CALCULATED* CONCENTRATION OF FREE NaF. (MGM. PER L.)
cc. sol. B in 280 cc.	Mgm. dried sub- stance per liter reaction mixture	Free Ens.	NaF. Ens.		
Amounts ap- proaching zero	Amounts ap- proaching zero	(52.6)	(47.4)	(0.90)	0.030 (total amount present)
5	5.7	53.	47.	0.89	0.030
10	11.4	52.	48.	0.93	0.029
25	28.6	55.	45.	0.82	0.027

* The last column gives a uniform series, although the figures in the next to the last column do not diminish regularly.

The experiment was successful in its primary purpose; *i.e.*, it showed that in mixtures of low enzyme concentration almost all the sodium fluoride was free, but failed in its secondary purpose; *i.e.*, it did not show how much sodium fluoride was combined with a given amount of enzyme.

One of the most important points about this reaction is that it is reversible. Loevenhart and Peirce⁷ mixed esterase and sodium fluoride and dialyzed the mixture. After dialysis the solution had regained its original activity.

The experiment was conclusive evidence for dissociation of the inactive compound, provided an inactive compound was formed under those conditions (*i.e.*, mixture of the enzyme with sodium fluoride). It is, however, possible, and indeed probable, that the presence of ethyl butyrate or alcohol or butyric acid or even two or three of these substances is necessary for the formation of the inactive compound.⁸ The evidence for the exact nature of this inactive compound will be presented in a succeeding paper, but the question does not concern us here. The reversibility of its formation is, however, easily demonstrated.

For instance, in a 250 cc. mixture containing 153.4 cc. $\frac{N}{20}$ ethyl butyrate, 10 cc. of enzyme and 1:6,000,000 sodium fluoride the action proceeded as if only 27.6 per cent of the enzyme present

⁷ This *Journal*, ii, p. 406, 1907.

⁸ For comment on these points, see Conclusion 7 at the end of this paper.

were acting. At a given time (fixed by a preliminary experiment) 5 cc. $\frac{N}{20}$ butyric acid had been produced per 50 cc., so that 50 cc. of the mixture then contained 5 cc. $\frac{N}{20}$ butyric acid, 5 cc. $\frac{N}{20}$ alcohol and 25.68 cc. $\frac{N}{20}$ ethyl butyrate. Fifty cc. of this solution were now added to 200 cc. of a mixture containing the same amount of ethyl butyrate, alcohol and butyric acid, but free from enzyme and sodium fluoride. In so doing, the enzyme and sodium fluoride were diluted five times, leaving all other factors unchanged. Two possibilities were now open for the further course of the reaction. In the first place, it might have proceeded one-fifth as fast as it did before dilution (where only 27.6 per cent of the enzyme was acting) or it might have produced acid at the same rate as a solution originally made up with 2 cc. enzyme in 250 cc. containing sodium fluoride 1:30,000,000. A control solution made up in this way worked as if about 59 per cent of the enzyme were acting, and corresponded to what was actually observed after dilution. We had, therefore, before dilution 27.6 per cent of the enzyme acting and 72.4 per cent combined with the sodium fluoride, whereas after dilution only about 41 per cent of the enzyme was present in the inactive form. The difference was great enough to be unmistakable, and gave good evidence for the fact that the reaction is reversible, whatever the nature of the inactive compound.

The data in the following experiment were obtained in the usual way, with two exceptions. First: the 50 cc. of solution to be titrated were run into 25 cc. of neutralized 80 per cent alcohol. This stopped the action more effectively than strong sodium fluoride. Second: 25 cc. instead of 50 cc. were in several instances used for a titration on account of lack of material. This accounts to a certain degree for divergence of the controls, as the titration errors must be multiplied by two.

A partial discussion of the results in the following table has just been given and the full data will now be presented.

TABLE V A.

200 cc. Ethyl butyrate solution (50 cc. = 38.35 cc. $\frac{N}{20}$ solution).
 10 cc. Enzyme solution C.
 25 cc. Sodium fluoride 1:600,000.
 15 cc. Water.

A (OBSERVED)		A (AVERAGE)		$\frac{A^*}{5}$ (CALCULATED)	
x (cc. $\frac{N}{20}$ acid)	t (min.)	x	t	$5t$	$5t-118.7$
2.01	13.08	2.11	13.94		
2.21	14.80				
3.68	29.70	3.74	30.29		
3.80	30.88				
		(4.88)	(43.7)	218.5	99.8
		(5.00)	(45.2)		
5.32	49.12	5.41	50.48	252.4	133.7
5.49	50.83				
8.85	98.43	8.94	100.01	500.0	381.3
9.03	100.58				
		(10.00)	(114.7)	573.5	454.8
10.34	120.08	10.43	120.78	603.9	485.2
10.52	121.67				

* For explanation of columns 5 and 6, see description of Figure 2.

A second similar solution was made up (also in duplicate), and at the end of approximately 44 minutes, 50 cc. of it were added to the following solution:

134 cc. Ethyl butyrate solution
 20 cc. $\frac{N}{20}$ Butyric acid.
 5 cc. $\frac{N}{5}$ Alcohol.
 41 cc. Water.

The first titration was made within 45 seconds of mixing and the time taken as 0 at this point.

TABLE V B.

OBSERVED		AVERAGE	
x	t	x	t
4.88	0.00	4.88	0.00
4.87	0.00		
5.39	14.62	5.44	14.85
5.49	15.08		
6.42	41.37	6.52	43.59
6.61	45.80		
7.36	74.75	7.50	75.65
7.64	76.55		
8.84	118.72	8.87	119.20
8.90	119.68		
10.42	163.80	10.28	164.63
10.14	165.45		

The two following experiments were also necessary:

TABLES V C AND V D.

C

D

200 cc. Ethyl butyrate solution.
 10 cc. Enzyme solution C.
 0 cc. Sodium fluoride.
 40 cc. Water

500 cc. Ethyl butyrate solution.
 5 cc. Enzyme solution C.
 12.5 cc. Sodium fluoride 1:600,000.
 107.5 cc. Water.

OBSERVED		AVERAGE		OBSERVED		AVERAGE	
x	t	x	t	x	t	x	t
4.79	12.25	4.91	12.76	2.50	42.28	2.46	41.41
5.03	13.27			2.41	41.53		
7.55	21.47	7.57	21.87	3.93	73.62	3.87	73.31
7.58	22.27			3.80	73.00		
						(4.88)	(99.8)
9.50	29.07	9.42	29.43	5.21	106.87	5.13	106.73
9.34	29.78			5.04	106.58		
		(10.00)	(31.6)				
10.92	35.20	10.69	34.70	6.28	135.47	6.17	135.61
10.45	34.21			6.05	135.75		
				7.53	175.58	7.39	175.29
				7.24	175.00		
				8.92	222.03	8.75	222.20
				8.57	222.37		
						(10.00)	(267.7)
				10.41	276.28	10.24	276.33
				10.06	276.38		

The results are also expressed graphically in the following diagram. The letters of the curves refer to the preceding table.

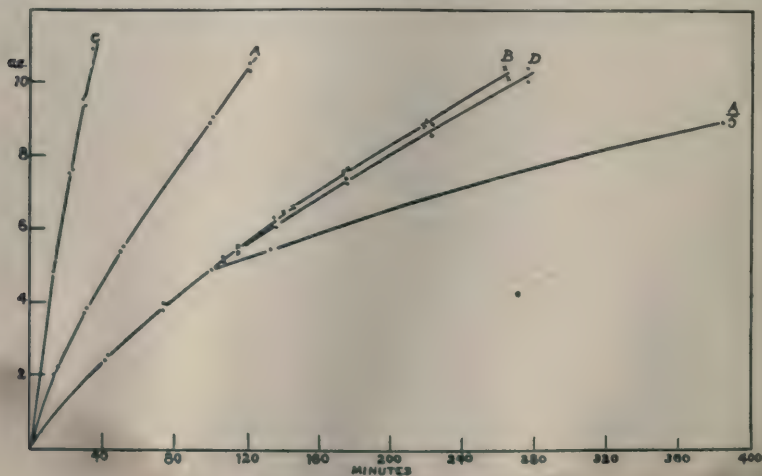


FIG. 2. TO ILLUSTRATE TABLE V.

$\frac{A}{5}$ represents a reaction going one-fifth as fast as A. A, C and D start from the origin. On the curve D, 4.88 cc. acid were produced in 99.8 minutes; but B, as observed, begins at $x = 4.88$ and $t = 0$. To make the points $x = 4.88$ on the two curves coincide, 99.8 is added to the values of t in plotting the curve B. In plotting $\frac{A}{5}$ the values of t , for curve A, are multiplied by 5. For $x = 4.88$, $t = 218.5$. In order to make the point $x = 4.88$ on this curve coincide with the corresponding points on B and D, 118.7 is subtracted from the values of t .

In spite of the apparent complexity of this experiment, the point that it makes is very simple. It shows that a given mixture of enzyme, sodium fluoride, ethyl butyrate, alcohol and butyric acid, if diluted five times, with the proper mixture of ethyl butyrate, alcohol and butyric acid, is *more than* one-fifth as active as it was before dilution. Since an enzyme solution that contained no fluoride would have been only one-fifth as active, the additional activity must have come from the partial dissociation of some sort of inactive compound present in the solution. This reversibility of the formation of the inactive compound absolutely excludes destruction of the enzyme by the sodium fluoride. In

addition, the fact that curves B and D so nearly coincide, shows that the point of equilibrium demanded by equation [3] is reached almost instantly from both directions.

CONCLUSIONS.

1. Sodium fluoride forms a compound with the esterase from pig's liver. This compound has little, if any, hydrolytic action on ethyl butyrate.

2. The formation of this compound is reversible.

3. When the concentration of the sodium fluoride is varied from 0.00893 mgm. per liter to 0.268 mgm. per liter, the inhibition increases from 20.8 per cent to 88.06 per cent.

4. Although theoretically we should expect a given amount of sodium fluoride to have less inhibiting effect in mixtures containing a large amount of enzyme, than in weaker enzyme mixtures, the difference actually found was very slight. This indicates that in the weaker mixtures, at least, very little of the total sodium fluoride present enters into the formation of the inactive compound.

5. The following equation, based on the supposition that one molecule of the inactive compound contains one molecule of enzyme and one molecule of sodium fluoride, agrees with the observations:

$$\text{Conc. free Enzyme} \times \text{Conc. free NaF} = k \text{ Conc. (NaF. Enz.)}$$

6. The observations will not agree with an equation based on any other supposition as to the number of molecules of sodium fluoride or enzyme entering into the formation of the inactive compound. For this reason it is justifiable to conclude for the present that one molecule of the inactive compound contains only one molecule of enzyme and one molecule of sodium fluoride.

7. It is possible that ethyl butyrate, alcohol or butyric acid are also constituents of the inactive compound. This does not affect the argument in any way. It is merely necessary to consider that the "free enzyme" of the above equation represents all that enzyme which is not contained in the inactive fluoride compound. According to a preceding paper,⁹ a large part of this

⁹ *Journ. Amer. Chem. Soc.*, **xxxi**, p. 1528, 1910.

"free enzyme" is present in the form of a compound with ethyl butyrate, so that only a part of the so-called "free enzyme" is actually free. Whether the sodium fluoride combines with some compound of the enzyme or with the enzyme actually free is immaterial, provided the experiments are so arranged that the concentration of the substance with which the sodium fluoride combines is proportional to some quantity that we know. This is done by never comparing any two solutions unless the concentrations of the ethyl butyrate, alcohol, butyric acid and hydrogen ion are the same at the stage of the reaction where the two solutions are compared. Under such conditions the concentrations of all enzyme compounds, except the fluoride compound, are proportional¹⁰ to the "free enzyme" of the equation, so that the mathematical treatment is justified.

¹⁰ This statement must be modified if any enzyme compound that contains two molecules of enzyme is present in large amounts. Practically, there is no evidence that such compounds occur, so that for a preliminary investigation such as the present, the possibility of their existence may be neglected.

THE RATE OF ELIMINATION OF NITROGEN AS INFLUENCED BY DIET FACTORS.¹

I. THE INFLUENCE OF THE TEXTURE OF THE DIET.

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INTRODUCTION.

The changing view as to the extent of digestion before absorption and the probability that proteins are split to amino-acids has raised the question whether all amino-acids are utilized alike. Are not some more resistant in metabolism than others? Does not deaminization take place with greater difficulty in some cases? If it does, one can easily conceive how different may be the behavior of the various proteins in nutrition. Bearing in mind the well-known fact that proteins vary widely in chemical composition, it is evident that the products of absorption after the ingestion of one protein may be much unlike those when another is fed. Thus, if the assumption that amino-acids are of variable resistance is correct, we may have an entirely different metabolic picture in the two cases.

A study of the rate of elimination of nitrogen² in the urine suggests itself as a means of ascertaining whether or not the amino-acids behave alike in metabolism. It is obvious that any variation in the ease of deaminization of the amino-acids may lead to a

¹ The experimental data embodied in the papers of this series are taken from the dissertation submitted by Robert C. Lewis for the degree of Doctor of Philosophy, Yale University, 1912.

² For a review of the literature on the rate of elimination of nitrogen see: Graffenberger: *Zeitschr. f. Biol.*, xxviii, p. 318, 1891; Hawk: *Amer. Journ. of Physiol.*, x, p. 115, 1903; Stauber: *Biochem. Zeitschr.*, xxv, p. 187, 1910; Wolf: *ibid.*, xl, p. 193, 1912.

change in the nitrogen-output curve after the ingestion of different amino-acids or of proteins of unlike composition. Other points, however, must be taken into consideration. Will a change in the rate of elimination of nitrogen necessarily be due to a difference in the metabolic behavior of the amino-acids? Certainly several other factors may play a part in this connection. Variations in the rate of the different processes of alimentation—gastric digestion, discharge of the food residues from the stomach and their passage along the digestive tract, pancreatic and intestinal digestion, absorption—may have a decided influence on the rapidity with which nitrogen leaves the body after a protein meal. Furthermore, metabolic processes distinct from deaminization, such as the behavior of the non-nitrogenous foodstuffs in influencing protein metabolism, are not without significance in this connection. All these factors must be considered as having a bearing in a study of the rate of elimination of nitrogen.

It seems quite probable that the lack of concordance in the nitrogen-output curves found by previous investigators may be due to a variation in certain incidental factors of the diet, such as the form in which the protein was taken, the amount of carbohydrate and fat ingested along with the protein, the water intake with the meal, and finally the proportion of indigestible material. Since the initiation of these investigations Benedickt and Roth³ have suggested comparable explanations for the discrepancies in the results of earlier workers.

That indigestible materials have an influence on alimentary processes is a familiar fact. Hedblom and Cannon⁴ have observed that coarse branny foods in the diet cause a more rapid discharge of the stomach contents. Recently Mendel and Fine⁵ have shown that indigestible substances added to the daily meal even in small quantities cause a poorer utilization of protein. It seems quite probable, then, that the rate of elimination of nitrogen in the urine may be affected by the texture of the diet.

³ Benedickt and Roth: *Zeitschr. f. klin. Med.*, lxxiv, p. 74, 1911.

⁴ Hedblom and Cannon: *Amer. Journ. Med. Sci.*, xxxviii, p. 1, 1909.

⁵ Mendel and Fine: *this Journal*, xi, p. 5, 1912.

METHODS.

The method employed in the present series of investigations for studying the rate of elimination of nitrogen in the urine has been to collect the urine at definite intervals after the ingestion of protein, to determine its content of nitrogen for the different periods, and to obtain from the results a curve of nitrogen output. Bitches were used as subjects of investigation, the urine being obtained by catheterization. The elimination of individuality was secured by the use of more than one animal for each type of experiment. In the present paper, however, it will be necessary to limit ourselves to the report of a single experiment illustrating each point. The dogs used are designated by a specific letter in the number of the experiment.

While a series of experiments was in progress the animal received each morning in a single meal a definite ration—the "Standard Diet." On experimental days the meal differed from this "Standard Diet" either by having something added to it or by having one (or more) of its constituents replaced. The same amount of nitrogen was always given, however; and in the replacement of non-nitrogenous constituents isodynamic quantities of some other foodstuff were substituted. Preceding an experiment there was always one day with the "Standard Diet" and generally there were two or three. Three of these preliminary days were introduced at the beginning of each series so that the animal might have plenty of time for adjustment to the new régime. Thus day after day throughout the whole series the food contained the same amount of nitrogen and, except in a few cases, approximately the same calorie value.

On an experimental day the animal was catheterized in the morning and fed fifteen minutes after the beginning of catheterization. To avoid possible secretory disturbances, the temperature of the well mixed food was always the same—20°C.—at the time of ingestion. Usually the animal ate the entire meal greedily. At times, however, the food had to be forced. In all cases feeding was complete in ten minutes or less. Collections of urine were made three, six, nine, twelve, fifteen and twenty-four hours after the beginning of the experiment. In some experiments a control specimen of urine was collected for an hourly period before the

commencement of the experiment; but in the majority of cases a collection for the twenty-fifth hour was made. Catheterization was planned so as to take exactly ten minutes, the expiration of that time being awaited where necessary, before the completion of the final washing. Nitrogen was determined by the Kjeldahl-Gunning method.

The "Standard Diet" was, of course, arbitrarily chosen. The only requirements in selecting such a ration were that it must furnish at least the minimal protein requisite and sufficient calories for the needs of the body. It seemed desirable, however, to use more than the minimal protein requirement in order to have a liberal output of nitrogen in the urine. The ration adopted consisted of meat,⁶ lard, sucrose, bone ash⁷ (5 grams), and water, with the addition in some cases of NaCl (2 grams). The water was calculated on a basis of the dry constituents, three times as much water being added as there was dry material. In other words the water was present in the whole ration in about the same proportion as it is found in meat. The sugar and lard were given in quantities approximately isodynamic to each other. The meal always contained 0.6 gram of nitrogen per kilo of body weight and furnished about 70 calories per kilo. The exact calorific value of the diets is not known because the fat content of the meat was not determined. By employing a fixed "Standard Diet" which was easy to duplicate, the rates of elimination of nitrogen under the various experimental conditions with the same and different animals were readily comparable.

Inasmuch as the addition of indigestible materials to the "Standard Diet" suggests itself as a means of studying the influence of the texture of the diet on the character of the nitrogen-output curve, mineral oil, vaseline, paraffin, filter paper, ground cork, agar-agar, bone ash, and sand were added in various experiments. There is little question that these materials pass through the intestine chemically unchanged. Bone ash may possibly be dissolved to some extent in the hydrochloric acid of the gastric juice. With respect to the mineral oil Bradley and Casser⁸ have reported

⁶ Preserved frozen, according to the method of Gies.

⁷ For the use of bone ash see Steele and Gies, *Amer. Journ. of Physiol.*, xx, p. 343, 1907.

⁸ Bradley and Casser: Proceedings of the American Society of Biological Chemists, December 1911, this *Journal*, xi, p. xx, 1912.

that an emulsified mixture of olive and petroleum oils fed by sound to a dog leads to absorption of both fat and hydrocarbon—a result not in accord with the later experience of Bloor.⁹ The indigestibility of the other materials used is well established.

An amount of water equivalent to three times the weight of the superimposed substance was also added with two-fold purpose in the cases of the water-absorbing materials: filter paper, cork, and agar-agar. In the first place, without this extra water the added material could not have been soaked up and well mixed with the food. Secondly, the amount of water carried out through the bowel when these substances were used was relatively very great; and, in order to insure against a large loss of water from the tissues, it was necessary to give an added amount of water with the meal.

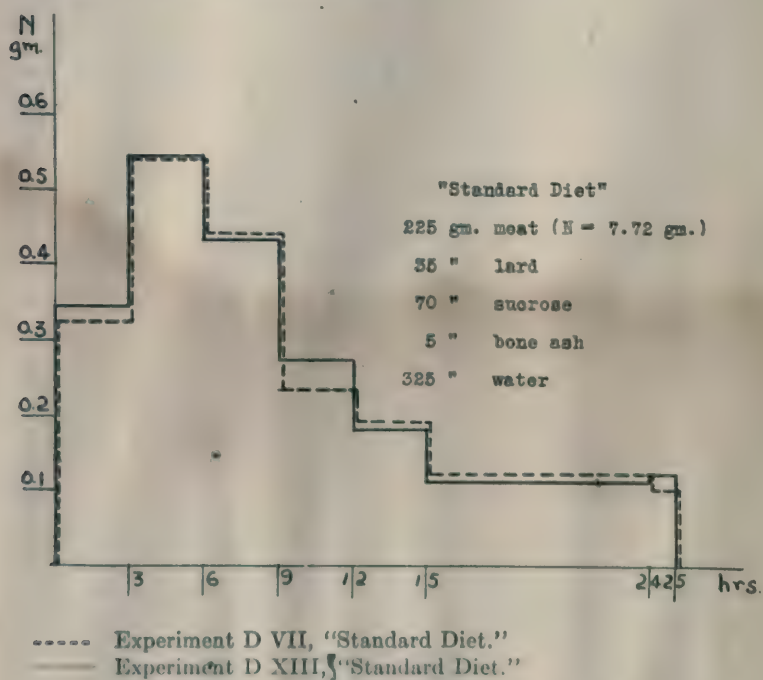
CONTROL EXPERIMENTS WITH THE “STANDARD DIET.”

Before attempting to determine the relation of the different diet factors to the rate of nitrogen elimination in the urine, it was necessary to ascertain the nature of the nitrogen-output curve after the ingestion of the “Standard Diet” and to see whether a characteristic curve always followed. In the text each type of experiment is illustrated by a curve, plotted from the data obtained. Curve I, a typical graphic illustration,¹⁰ shows the agreement of the nitrogen-output curves of two experiments with the “Standard Diet.” The abscissae represent equal increments of time; the ordinates, grams of nitrogen. Thus at a glance the average *hourly* rate of elimination of nitrogen for a single period is shown by the value of the ordinate. It is readily seen that after the ingestion of the “Standard Diet” there is a rise in the nitrogen output during the first period, reaching a maximum in the second three hours, followed by a fall to the initial level early the next morning. In the present work it has always been possible with the same animal to get “standard” experiments which agree within reasonably close limits (Curve I). Furthermore “standard” curves of duplicate character have been obtained repeatedly with different animals.

⁹ Bloor: *This Journal*, xv, p. 105, 1913.

¹⁰ All curves show the rate of nitrogen output in two experiments, a “standard” experiment (broken line) being plotted for purposes of comparison.

CURVE I. To illustrate the agreement of duplicate experiments after the ingestion of the "Standard Diet."

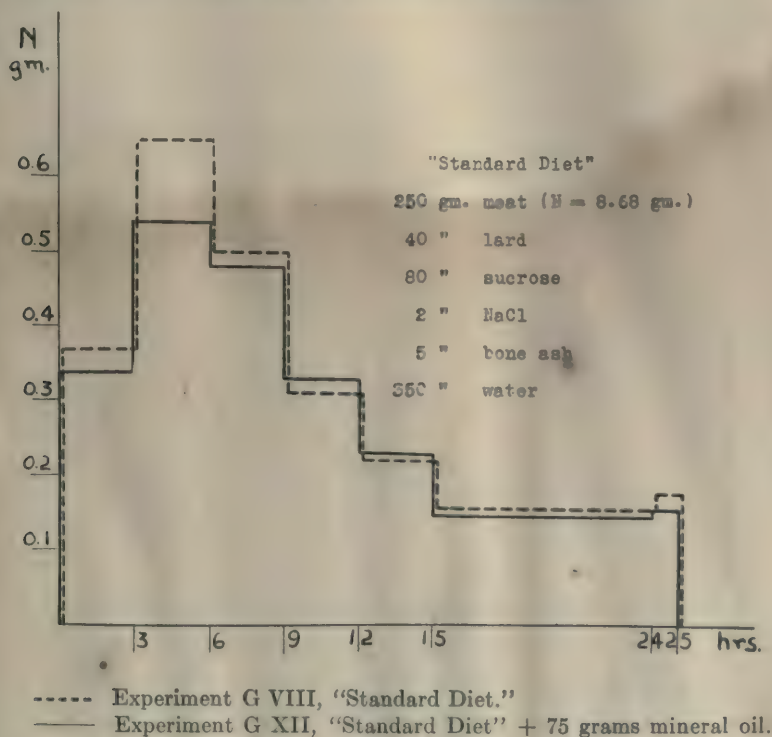


EXPERIMENTS WITH INDIGESTIBLE MATERIALS.

*Mineral oil*¹¹ (Curve II).

When mineral oil was added to the diet the nitrogen output in the second period was notably less than in the corresponding period after the ingestion of the "Standard Diet" alone. Evidently mineral oil causes a slower rate of elimination of nitrogen.

CURVE II. To illustrate the effect of an addition of *mineral oil* to the "Standard Diet" on the rate of elimination of nitrogen.

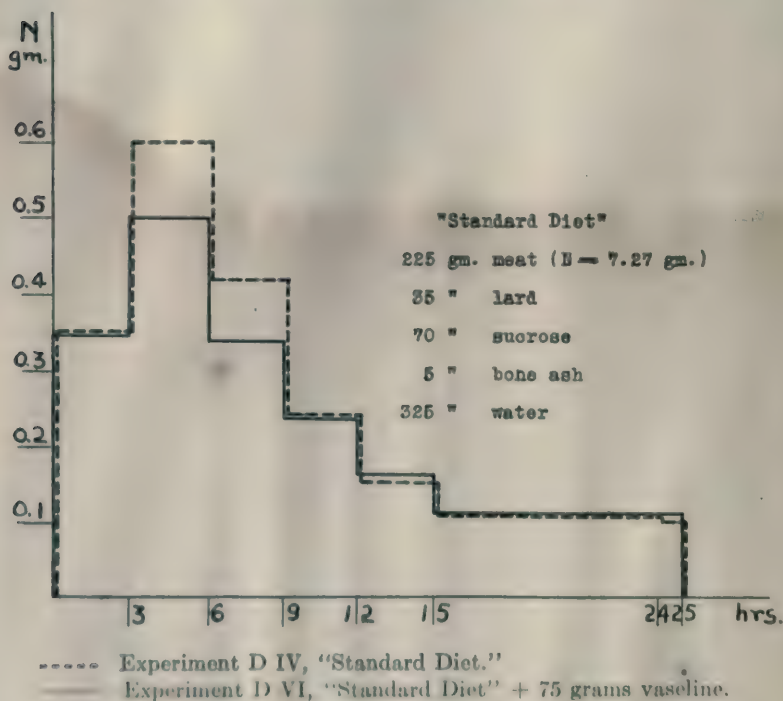


¹¹ A colorless, purified product sold under the trade name of "Alboline."

*Vaseline*¹² (Curve III).

The effect of vaseline on the curve of nitrogen elimination is similar to, but more marked than that of mineral oil. The nitrogen output in each of the first three periods is smaller than in the "standard" experiment; afterwards the two curves are almost identical. There is a delay in the excretion of nitrogen.

CURVE III. To illustrate the effect of an addition of *vaseline* to the "Standard Diet" on the rate of elimination of nitrogen.

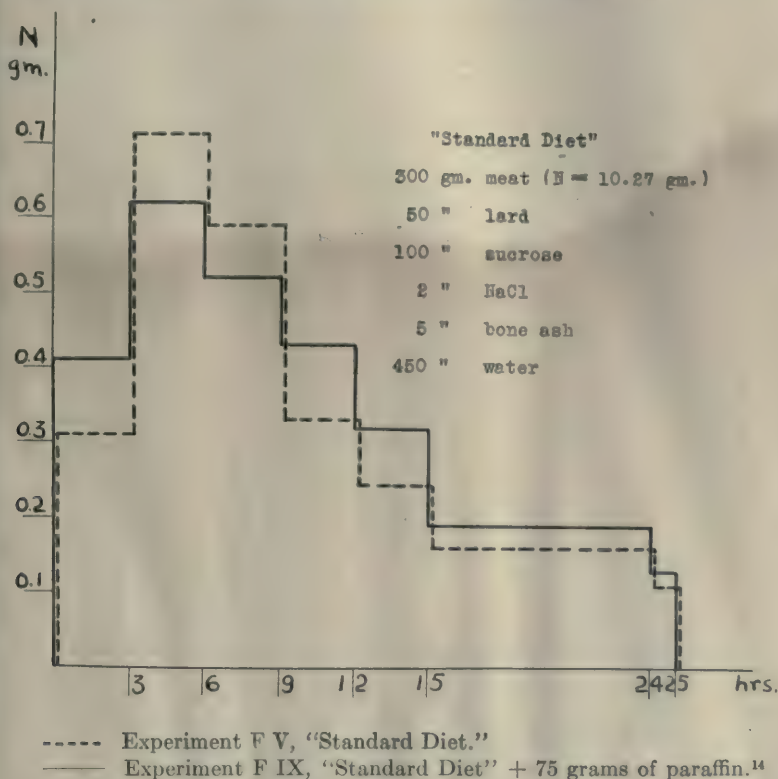


¹² Yellow petroleum jelly (M.P. = 38°C.).

*Paraffin*¹³ (Curve IV).

The experiment with paraffin shows a much more decided flattening of the nitrogen-output curve, *i.e.*, a preliminary* delay in excretion of nitrogen, than was the case with either of the softer petroleum products. The rate of elimination of nitrogen is not only lower in the earlier periods than in the "standard" experiment, but also higher during the latter part of the day.

CURVE IV. To illustrate the effect of an addition of *paraffin* to the "Standard Diet" on the rate of elimination of nitrogen.



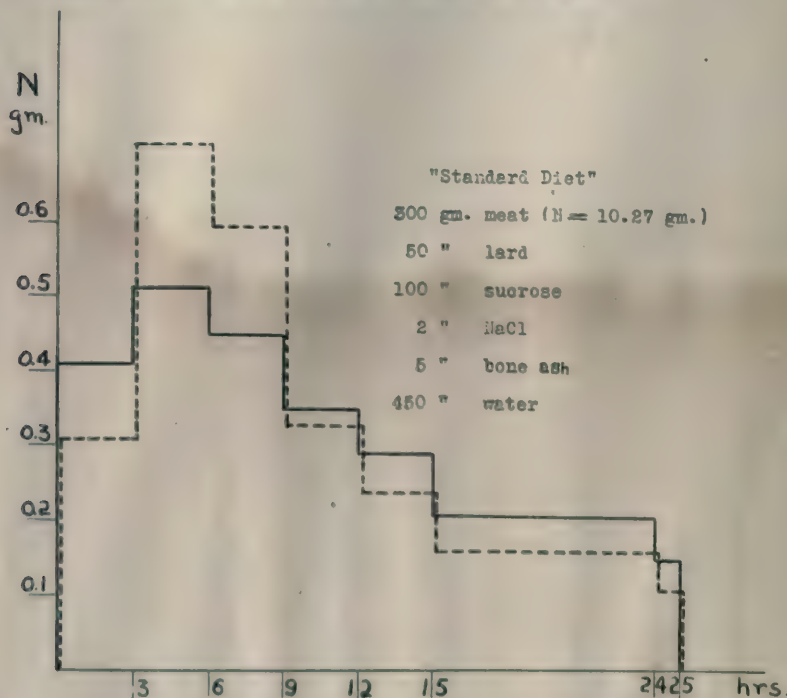
¹³ Fine shavings, obtained by scraping a cake of paraffin (M.P. = 51°C.) with a knife.

¹⁴ Large quantity of paraffin feces during the night (15-24 hour) period.

*Filter Paper*¹⁵ (Curve V).

The rate of elimination of nitrogen during the earlier periods after the ingestion of the "Standard Diet" plus filter paper is lower than in the "standard" experiment; during the later hours it is higher than normal. Thus, as was the case with paraffin, there results a very decided flattening of the nitrogen-output curve.

CURVE V. To illustrate the effect of an addition of *filter paper* to the "Standard Diet" on the rate of elimination of nitrogen.



----- Experiment F V, "Standard Diet."

———— Experiment F VIII, "Standard Diet" + 75 grams filter paper¹⁶ and 225 grams water.

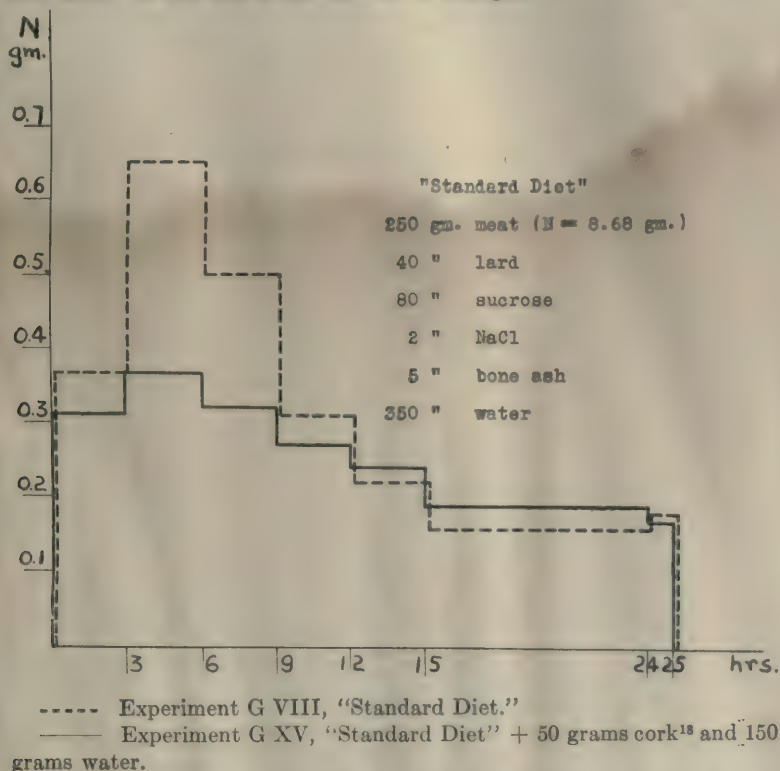
¹⁵ Cut up in small pieces.

¹⁶ Large quantities of paper feces during the 4th and 5th three-hour periods and during the night (nine-hour) period.

*Cork*¹⁷ (Curve VI).

With cork there is likewise a much slower elimination of nitrogen than with the "Standard Diet" alone. In this case the total nitrogen output for the entire day is lower than in the "standard" experiment. This, however, is not the only cause of the sub-normal elimination of nitrogen in the *early periods* of the day; for the character of the nitrogen-output curve is radically different from the "standard"—lower during the early periods and higher during the later periods of the day.

CURVE VI. To illustrate the effect of an addition of *cork* to the "Standard Diet" on the rate of elimination of nitrogen.



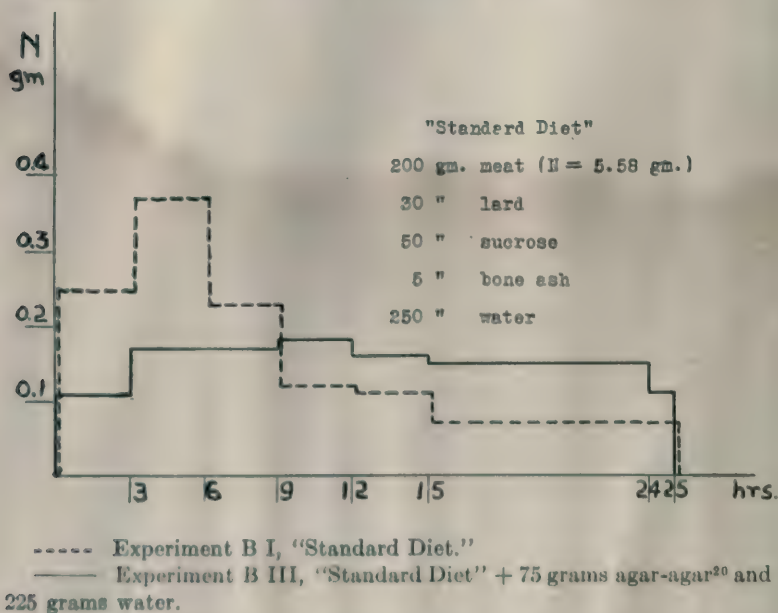
¹⁷ Finely ground in a coffee mill.

¹⁸ Large quantities of cork feces during the 4th and 5th three-hour periods and during the night (nine-hour) period.

*Agar-agar*¹⁹ (Curve VII).

Of all the indigestible materials used the agar-agar caused the most pronounced delay in the nitrogen output. In the experiment here reported there was a rise in the second period over the value of the first three hours and then very little change for twelve hours; in other experiments there was a similar though slightly less marked effect.

CURVE VII. To illustrate the effect of an addition of *agar-agar* to the "Standard Diet" on the rate of elimination of nitrogen.



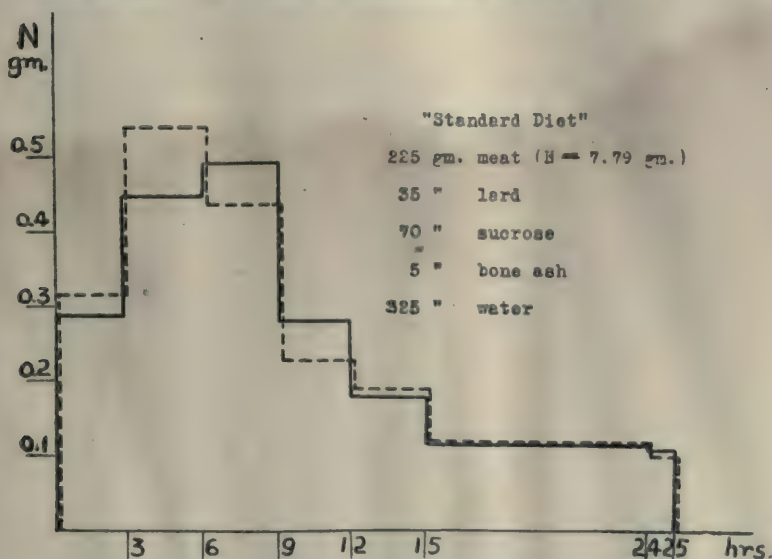
¹⁹ Very finely chopped.

²⁰ First agar-agar feces during first three-hour period. No note kept of subsequent defecations; feces in almost every period, however.

Bone Ash (Curve VIII).

With the addition of bone ash to the "Standard Diet" there is a flattening of the curve, but by no means to such an extent as with any of the previously mentioned indigestible materials except the softer petroleum products. There is a delayed excretion of nitrogen in the first two periods, followed by a slight compensatory rise during the next six hours, the curve afterward running parallel to that of the "standard" experiment.

CURVE VIII. To illustrate the effect of an addition of *bone ash* to the "Standard Diet" on the rate of elimination of nitrogen.



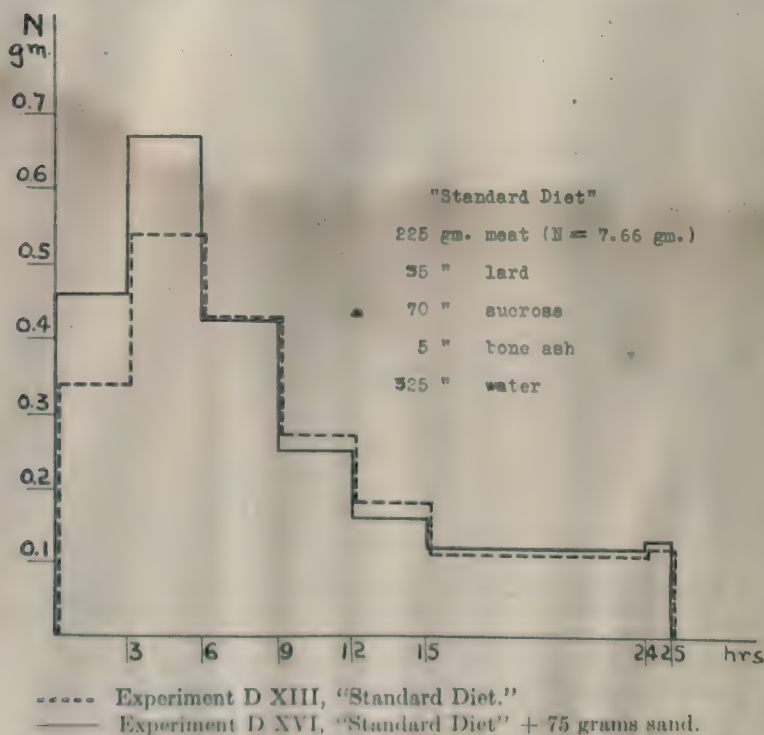
----- Experiment D VII, "Standard Diet."

— Experiment D IX, "Standard Diet" + 75 grams bone ash.

Sand (Curve IX).

The effect on the nitrogen-output curve of an addition of very fine sand to the "Standard Diet" is entirely different from anything so far reported. The rate of elimination of nitrogen during the first two periods is notably *higher* than in the corresponding periods of the "standard" experiment; afterward the two curves run parallel.

CURVE IX. To illustrate the effect of an addition of *sand* to the "Standard Diet" on the rate of elimination of nitrogen.



DISCUSSION.

The experiments with a variety of indigestible materials have shown a slower rate of elimination of nitrogen after the addition of these substances to the "Standard Diet" except in the case of sand. Obviously there has been some delay in the processes of alimentation; for, excepting differences in the amount of water absorbed,²¹ the purely metabolic conditions are the same when an indigestible substance is included in the daily meal as when the "Standard Diet" alone is fed. The prime factor in this delay must have been a slower rate of absorption, whether induced by a retardation of the discharge of the gastric contents, a delay in digestion, an adsorption of digestive products by the indigestible material, or a loss of absorbable material by an early evacuation of the bowel. Let us consider the bearing of each of these contributory factors on the present work.

In all probability no delay in the *discharge from the stomach* occurred when indigestible materials were added to the diet. With mineral oil, vaseline, paraffin, and bone ash the passage of food onward must have been as rapid as under normal conditions; for during the first periods with these materials there was no marked decrease in the nitrogen output below that of the "standard" experiment. The early appearance of the added indigestible material in the feces following the ingestion of filter paper, cork, and agar-agar²² suggests an acceleration rather than a retardation of the normal gastric discharge. This is in harmony with the report of Hedblom and Cannon²³ that branny foods cause a more rapid emptying of the stomach.

A delayed absorption on account of a sub-normal *rate of digestion* in the experiments with indigestible materials is quite possible.

²¹ With several of the indigestible substances—cork, filter paper, and agar-agar—a large amount of water was excreted through the bowel. As a result the volume of urine, and hence the water absorbed, was very small. That the lack of water was not an important factor in causing a retardation of the rate of elimination of nitrogen has been shown by an experiment with agar-agar in which a very large amount of water was given. In this case there was a normal flow of urine, but the same effect was obtained as when the secretion of urine was small.

²² The influence of these substances on the emptying of the bowel is indicated in the reports of the experiments.

²³ Hedblom and Cannon: *Amer. Journ. Med. Sci.*, cxxxviii, p. 1, 1909.

It was pointed out in the preceding paragraph that some of the indigestible materials caused a more rapid discharge from the stomach. This might very well lead to a slower rate of digestion because the preliminary gastric proteolysis was inhibited before it had advanced very far, and the intestine thus received much more complex residues than under normal conditions. This possibility of an unfavorable result from sparing the stomach at the expense of the intestine has been previously mentioned by Cohnheim²⁴ and Cannon.²⁵ It is interesting to note that it was in the cases where there was an early emptying of the stomach that the greatest delay in elimination of nitrogen, and hence in absorption, occurred. Furthermore, digestion may have been delayed because the food residues, mixed as they must have been with the indigestible materials, are rendered more or less inaccessible to the action of the digestive enzymes.

Another possible explanation for the delay in absorption when indigestible substances are added to the diet is that the *products* of digestion are *adsorbed* by the added indigestible material. It is quite conceivable that agar-agar, for example, might adsorb the soluble intestinal contents. An examination of the data shows that the indigestible materials exerted a retarding influence on the rate of absorption, as measured by the rate of elimination of nitrogen. This retardation was progressively greater in the following order—mineral oil, vaseline, bone ash, paraffin, filter paper, cork, agar-agar—corresponding with the comparative adsorptive power of the same substances. This parallelism between delayed absorption and power of adsorption seems to be more than a coincidence. Adsorption of intestinal contents by the indigestible adjuncts might cause a slower rate of absorption in two ways: in the first place, the partially digested protein residues might in part be rendered less accessible to enzyme action; secondly, a smaller proportion of the completely digested protein residues would come in contact with the intestinal wall, and for this reason absorption would be hindered.

It is unlikely that the delayed absorption in the experiments with indigestible materials is attributable to an early *emptying*

²⁴ Cohnheim: Quoted by Cannon.

²⁵ Cannon: *The Mechanical Factors of Digestion*, International Medical Monographs I (Longmans, Green and Company), 1911, p. 123.

of the bowel and a consequent loss of available nitrogen. The more than compensatory rise in the urinary nitrogen output above the normal which always occurs in the later periods with the substances causing early evacuation makes such an explanation questionable.

There remains a consideration of the results obtained with sand. The data presented emphasize an increased elimination of nitrogen in the first periods after the meal when sand is added to the "Standard Diet." Can this rise be caused by a more rapid discharge of the gastric contents? No evidence has been found in the literature to warrant such a conclusion. On the contrary the results of Hedblom and Cannon,²⁶ showing that hard irregular pieces of dried starch paste in the diet caused a slower discharge of the stomach, appear to speak against such an explanation. It is unlikely that sand has mechanically stimulated an increased secretion, the reabsorption²⁷ of which has raised the nitrogen output of these first two periods; for Pawlow²⁸ has demonstrated conclusively that the blowing of sand with force against the walls of the inactive stomach does not stimulate gastric secretion. The failure of sand to stimulate secretion is further shown by the following experiment:

During the middle of the fourth day of a fast the urine was collected for a three-hour control period. A quantity of sand (and a little water) was then given; and the urine was subsequently collected at three-hour intervals for nine hours. There was no increase in the nitrogen output of these later periods over that of the control period.²⁹

²⁶ Hedblom and Cannon: *Amer. Journ. Med. Sci.*, cxxxviii, p. 1, 1909.

²⁷ Mosenthal (*Journ. Exp. Med.*, xiii, p. 319, 1911) has attributed to the intestinal secretion a considerable source of absorbable nitrogen. From experiments on dogs with isolated loops of intestine he estimated that the nitrogen content of the succus entericus secreted in twenty-four hours was equivalent to about 35 per cent of the nitrogen intake. Inasmuch as the feces contained nitrogen equivalent to only 10 per cent of the intake, the major part of the intestinal secretion must have been reabsorbed.

²⁸ Pawlow: *The Work of the Digestive Glands*, translated by W. H. Thompson (Charles Griffin and Company), 1902, pp. 86-90.

²⁹ In a previous experiment we had ascertained that the nitrogen-output curve during starvation varied but little from a straight line.

SUMMARY.

The typical curve of nitrogen elimination on a selected mixed diet shows a rise in the first period, reaching a maximum in the second three hours, followed by a fall to the initial level early the next day.

With a definite diet it has always been possible to duplicate experiments on the same animal. Different animals on the same type of diet have given parallel curves.

A delay in the elimination of nitrogen is caused by the addition to the diet of such indigestible materials as mineral oil, vaseline, bone ash, paraffin, filter paper, cork, and agar-agar—substances which act in a purely mechanical as contrasted with a chemical manner. Invariably there is a subnormal rate of nitrogen output in the first periods following ingestion of the meal; with paraffin, filter paper, cork, and agar-agar this is followed by a higher rate in the later periods. The effect of the indigestible materials is progressively greater in the order in which they are given above.

A delayed absorption of the nitrogen intake is presumed responsible for the slower rate of elimination of nitrogen. As possible causes of this retardation of absorption the following have been suggested: (1) a slower rate of digestion caused by an early emptying of the stomach and a consequent early exclusion of gastric proteolysis, with the possibility of a more prolonged intestinal digestion; (2) a slower rate of digestion caused by an adsorption of partially digested protein residues by the added indigestible material, making them less readily accessible to the action of the digestive enzymes; (3) an adsorption of the final digestive products by the indigestible substance whereby their absorption from the intestine is hindered.

Sand gives an exception to the results obtained with the other indigestible materials studied, as it causes an elimination of nitrogen above the normal in the first six hours. This rise is presumably not caused by an increased secretion and subsequent reabsorption of digestive juices, for the ingestion of sand during starvation has no effect on the nitrogen-output curve.

THE RATE OF ELIMINATION OF NITROGEN AS INFLUENCED BY DIET FACTORS.

II. THE INFLUENCE OF CARBOHYDRATES AND FATS IN THE DIET.

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New Haven, Connecticut.)

(Received for publication, August 4, 1913.)

Although the general effect of carbohydrates in the diet on the rate of elimination of nitrogen in the urine seems to be well established, no study of the comparative behavior of different carbohydrates has been attempted. In the past the method of investigation employed has been to superimpose the carbohydrate to be studied on a "standard" diet, to determine the rate of elimination of nitrogen after this augmented meal, and to ascertain the effect of the carbohydrate on the nitrogen-output curve by contrast with the rate when the "standard" diet alone was fed. The objection to this method is that on the day when carbohydrate is given the diet has a greater calorific value than the "standard" diet, so that the conditions on the two experimental days are not comparable from the standpoint of energy intake. In the present investigation¹ an isodynamic quantity of carbohydrate was substituted for the non-nitrogenous constituents of the "Standard Diet," and thus the calorie value of the food of all days remained the same. The following carbohydrates were chosen for study: the polysaccharides, starch and soluble starch; the disaccharide, sucrose; and the monosaccharide, dextrose.

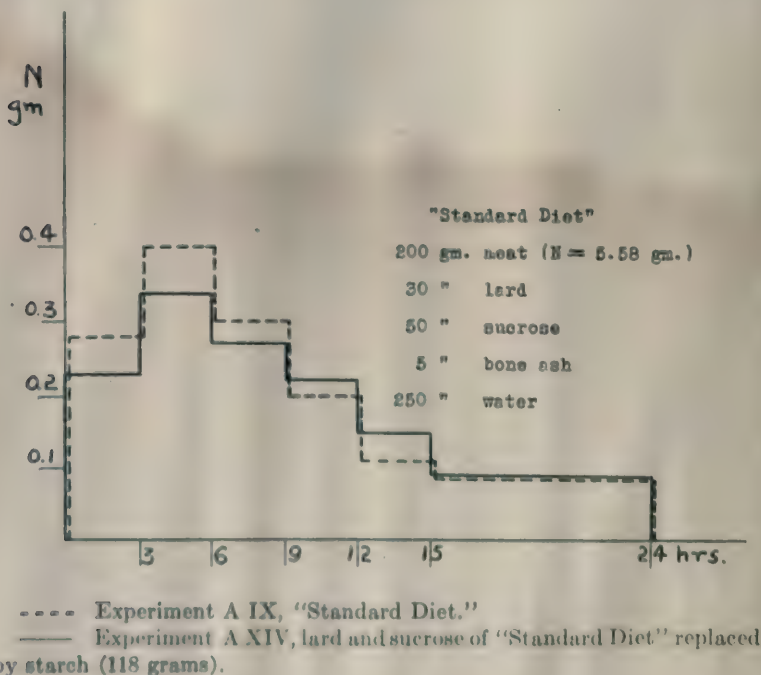
¹ The methods employed were those outlined in our first paper (cf. this *Journal*, xvi, p. 19, 1913).

EXPERIMENTS WITH CARBOHYDRATES.

*Starch*² (Curve I).

When starch was substituted for the non-nitrogenous constituents of the "Standard Diet" there was a distinct delay in the elimination of nitrogen, the amount of nitrogen excreted being smaller than in the "standard" experiment in the earlier periods of the day and larger in the later periods.

CURVE I. To illustrate the effect on the rate of nitrogen elimination of substituting *starch* for the non-nitrogenous constituents of the "Standard Diet."

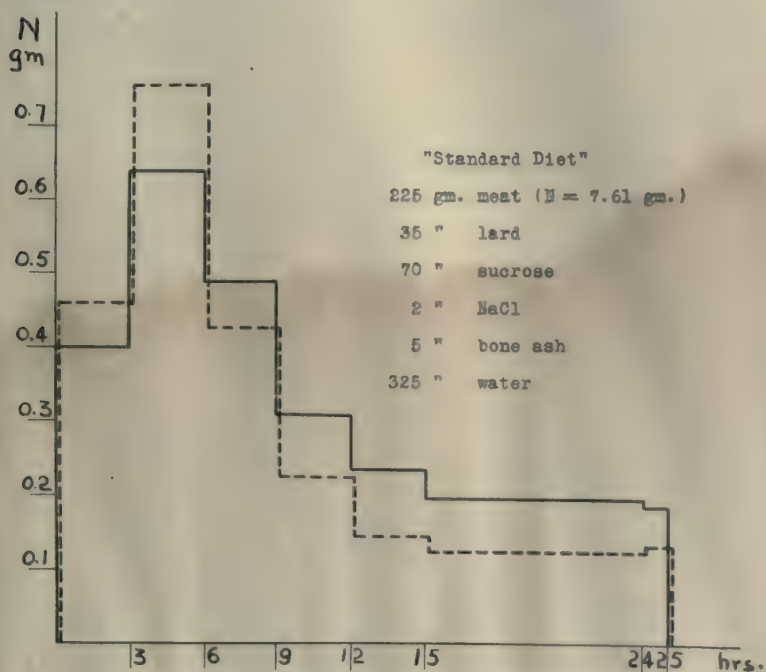


² Arrowroot starch was mixed with water and heated in an autoclave for fifteen minutes in order to rupture the starch grains.

Soluble starch (Curve II).

With the use of soluble starch in place of starch the retarding effect on the nitrogen excretion was even more marked.

CURVE II. To illustrate the effect on the rate of nitrogen elimination of substituting *soluble starch* for the non-nitrogenous constituents of the "Standard Diet."



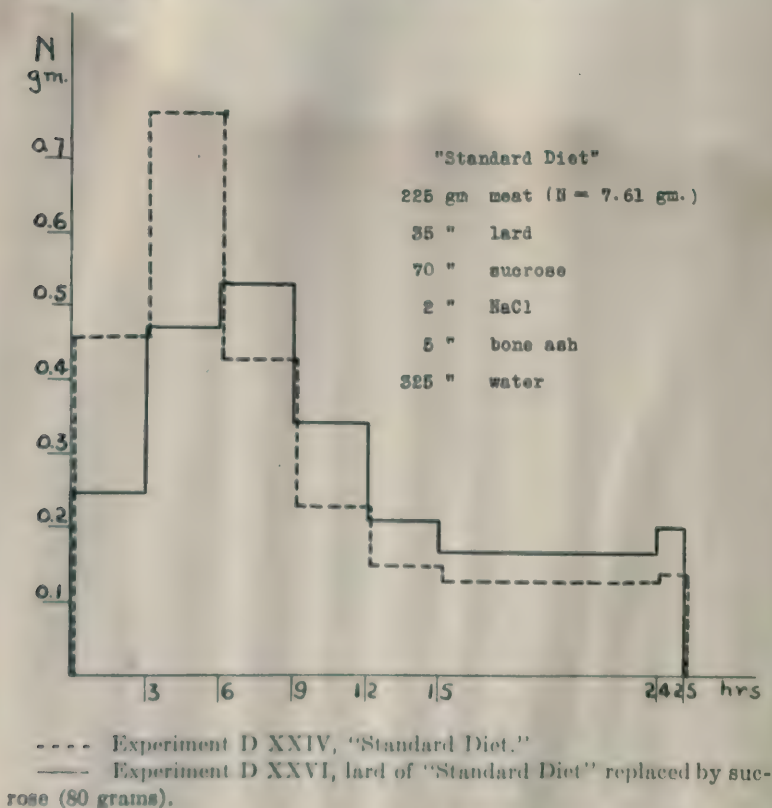
---- Experiment D XXIV, "Standard Diet."

— Experiment D XXVII, lard and sucrose of "Standard Diet" replaced by soluble starch (150 grams).

Sucrose (Curve III).

When sucrose was substituted for the lard of the "Standard Diet" the maximum output of nitrogen did not occur until the third three-hour period, as contrasted with the second period in the "standard" experiment and in experiments with the carbohydrates above reported. The nitrogen output was relatively much smaller in the earlier periods of the day and much larger in the later periods than was the case with either of the polysaccharides. In other words the flattening effect of sucrose on the nitrogen-output curve was much more pronounced than that of starch or of soluble starch.

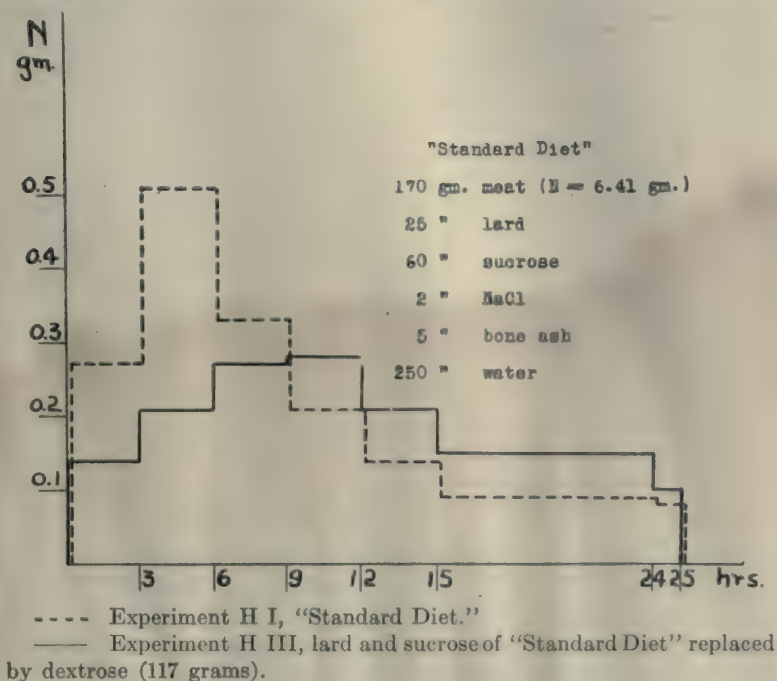
CURVE II. To illustrate the effect on the rate of nitrogen elimination of substituting *sucrose* for the lard of the "Standard Diet."



Dextrose (Curve IV).

With dextrose replacing the non-nitrogenous constituents of the "Standard Diet" the preliminary delay in nitrogen excretion was much greater than with any of the other carbohydrates studied, the nitrogen-output curve having a much more flattened aspect.

CURVE IV. To illustrate the effect on the rate of nitrogen elimination of substituting *dextrose* for the non-nitrogenous constituents of the "Standard Diet."



Sucrose; Lard being present in the diet.

In the experiments above reported the diets were fat-free and in this respect not comparable with the "Standard Diet." What effect would an increased amount of sucrose, for example, have on the rate of nitrogen elimination if lard were also present in the diet? In a further experiment the meal contained an amount of sucrose isodynamic to the non-nitrogenous constituents of the

"Standard Diet" and enough fat (94 grams of lard) to make the ratio of sugar to fat approximately the same as in the "Standard Diet." With such a procedure an increased number of calories was given, but to accomplish the desired end this was necessary. There was the same preliminary delay in the nitrogen excretion in this case with sucrose as when no fat was present in the diet.

DISCUSSION.

The substitution of the different carbohydrates for the non-nitrogenous constituents of the "Standard Diet" resulted in a slower rate of elimination of nitrogen, a flattening of the nitrogen-output curve. The carbohydrates studied were progressively more effective in the following order: starch, soluble starch, sucrose, and dextrose.

With the method employed in the present study with carbohydrates two important changes have been made in the diet: (a) fat has been removed; (b) an added amount of carbohydrate has been given. Which of these two changes is of paramount importance in causing the results above reported? An experiment in which fat as well as sucrose was added to the "Standard Diet" shows that the removal of the fat was not the causal factor; for here, as in the cases where sucrose replaced lard, the curve of nitrogen elimination is considerably flattened despite the presence of an abundance of fat. The added carbohydrate must have been directly responsible, then, for the slower rate of nitrogen elimination.

The results of the present investigation with carbohydrates are completely in harmony with those of previous investigators. Vogt (1906) found that the addition of rice or rice flour to a meat diet caused a slower rate of elimination of nitrogen than meat alone. Levene and Kober (1909) reported that with the addition of starch to a "standard" diet containing plasmon, cracker meal, and lard the course of nitrogen elimination did "not differ materially from that of the standard diet." A careful examination of the data presented by these authors discloses, however, a slight flattening of the nitrogen-output curve when starch was added to the diet. According to Van Slyke and White (1911) starch superimposed on a diet of fish, cracker meal, and lard caused a delayed elimination of nitrogen of about the same magnitude as in the starch experiments of the present work. Falta and Gigon (1908)

found a delayed excretion of nitrogen after the addition of either wheat flour or levulose to a meat diet, the effect being more marked in the case of the latter carbohydrate. This result with levulose agrees with that of Falta, Grote, and Staehelin (1907). Pari (1908) reported that with the addition of sucrose to a meat diet there was a retardation of the nitrogen excretion. Interesting in this connection are the experiments of Boettcher and Vogt (1909) in which subcutaneous injections of dextrose (5-10 grams) caused a flattening of the nitrogen-output curve. All of these investigators worked with dogs.³ Lusk (1912) fed dextrose alone to dogs 24 hours after the last meal and found a nitrogen output lower than the fasting level during the hour following the dextrose intake. Subsequently there was a compensatory rise in the nitrogen elimination.

Concerning the manner in which carbohydrate may be responsible for a delay in nitrogen excretion several possibilities must be considered, viz: a subnormal rate of discharge of the stomach contents, a retardation of digestion, a delayed absorption, altered metabolic processes. Van Slyke and White (1911) have given no experimental proof for their conclusions that the retardation of nitrogen elimination when starch is added to the diet is caused by a delay in digestion and absorption. The experiments of Boettcher and Vogt (1909), showing a delay in absorption after subcutaneous or intravenous dextrose injections in five out of seven cases, are hardly comparable with the experiments of the present series where the carbohydrate was given *per os*. In fact, no conclusive evidence has been found in the literature to the effect that a subnormal rate of any of the alimentary processes is caused by an addition of carbohydrate to the diet. On the contrary, the report of Cannon⁴ that a mixture of carbohydrate and protein foods leaves the stomach more rapidly than protein alone, whereas fat has a retarding action on the emptying of the stomach, makes it probable that the addition of carbohydrate to, and the removal of fat from the diet in the present experiments is, if anything, followed by a more rapid discharge of the gastric contents than in the "standard" experiment.

³ Wolf (1912) studied the rate of elimination of nitrogen after the ingestion of starch by a fasting man. The results have no bearing on the experiments here reported.

⁴ Cannon: *Amer. Journ. of Physiol.*, xii, p. 387, 1904.

There is some evidence in the literature that variations in metabolic processes are responsible for the slower rate of nitrogen elimination under the influence of carbohydrates. Falta and Gigon (1908) and Par. (1908) have attributed this delay to the protein-sparing action of carbohydrates; for after a fast, when the glycogen depots are almost depleted, the carbohydrates no longer exert a retarding action on the nitrogen-output curve. The reason for this, according to these authors, is that the carbohydrates now go to make up the depleted glycogen supply in preference to being immediately burned. Boettcher and Vogt (1909) think that a disturbance of intermediary metabolism is in part responsible for the delay in nitrogen excretion obtained after subcutaneous dextrose injections, although they offer no experimental proof for their contention. The consensus of opinion, then, seems to favor a disturbance of metabolic processes, rather than a delay in alimentation, as the causal factor in the retardation of nitrogen excretion when carbohydrate is present in the diet.

Although the experimental data obtained in this study do not warrant the adoption of a final theory as to how the carbohydrates act to retard nitrogen excretion, the writer is inclined to the belief that the protein-sparing action of carbohydrate causes this delay. When carbohydrate is present in the diet, it is digested, absorbed, and burned; while the protein residues, which are simultaneously absorbed, are temporarily spared to some extent and are only completely metabolized when carbohydrate is no longer available, a preliminary delay in nitrogen excretion thus occurring. If such a theory holds, the physiological, six-carbon sugar dextrose should be more efficient than the polysaccharide starch in causing a retardation of nitrogen excretion; for the more nearly the carbohydrate is prepared for absorption when ingested, the sooner should its sparing action come to expression, and so the greater should be the delay in nitrogen elimination. As a matter of fact the carbohydrates studied did show a progressively greater retarding effect in the order: starch, soluble starch, sucrose, dextrose. Thus the theory accounts for all the results obtained.

In conclusion reference should be made to the effect of indigestible carbohydrates in the diet on the rate of elimination of nitrogen. In the previous paper⁵ it was shown that cellulose—filter paper

⁵ Mendel and Lewis: *This Journal*, xvi, p. 19, 1913.

and cork—and agar-agar caused a delay in nitrogen excretion. It is obvious, however, that the explanations of the similar effect on the rate of nitrogen elimination after the ingestion of digestible and indigestible carbohydrates, respectively, are radically different.

SUMMARY OF RESULTS WITH CARBOHYDRATES.

Carbohydrates in the diet cause a slower rate of elimination of nitrogen after a protein meal, the various carbohydrates studied having a progressively greater effect in the following order: starch, soluble starch, sucrose, dextrose.

The experimental data do not warrant the adoption of more than a tentative theory as to the explanation of the retardation of nitrogen excretion when carbohydrates are present in the diet. It seems quite probable, however, that the protein-sparing action of carbohydrate is responsible for this delay. At any rate all the results obtained in the experiments with carbohydrates may be explained by such a theory.

THE RELATION OF FATS IN THE DIET TO THE RATE OF ELIMINATION OF NITROGEN.

No comparative study of fats of different texture—soft or hard—has been attempted in the few previous investigations of the influence of fat in the diet on the rate of nitrogen elimination. Most of the work in the past has been done by superimposing the fat on a “standard” diet, and determining its effect on the nitrogen-output curve. Inasmuch as this procedure is open to the objection that with the addition of fat to the diet an increased number of calories is given, a method similar to that employed in the study of carbohydrates⁶ was adopted for fats, the non-nitrogenous constituents of the “Standard Diet” being replaced by the fat to be studied. The following fats of widely different textures were used: cotton-seed oil, lard, and “Oleo-stearin”⁷ (M.P. = 53°C.).

⁶ See the first part of this paper.

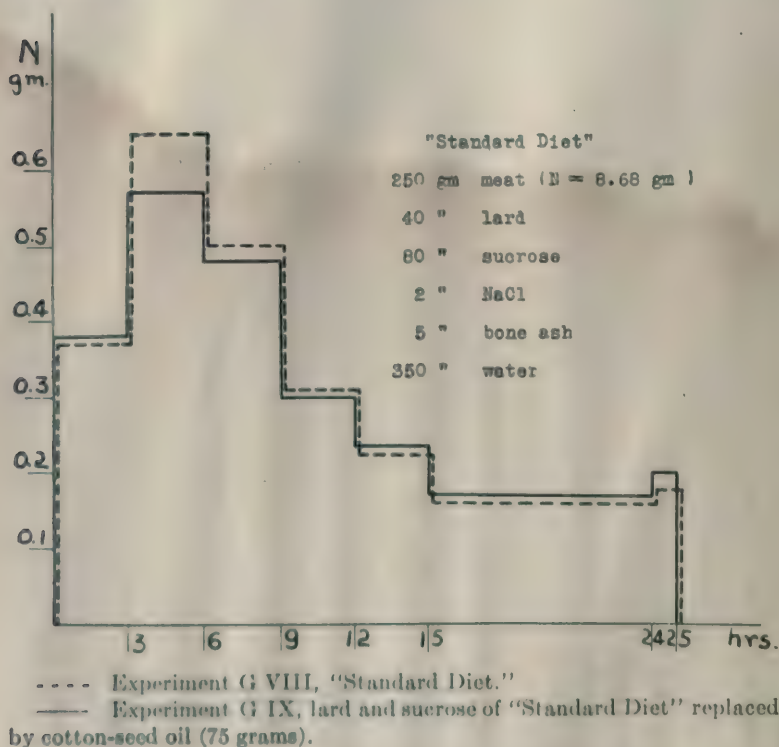
⁷ Armour and Company kindly furnished this product.

EXPERIMENTS WITH FATS.

Cotton-seed oil (Curve V).

The substitution of cotton-seed oil for the non-nitrogenous constituents of the "Standard Diet" had very little effect on the course of the nitrogen-output curve, the only variation from the "standard" occurring in the second three-hour period where the nitrogen output was decreased.

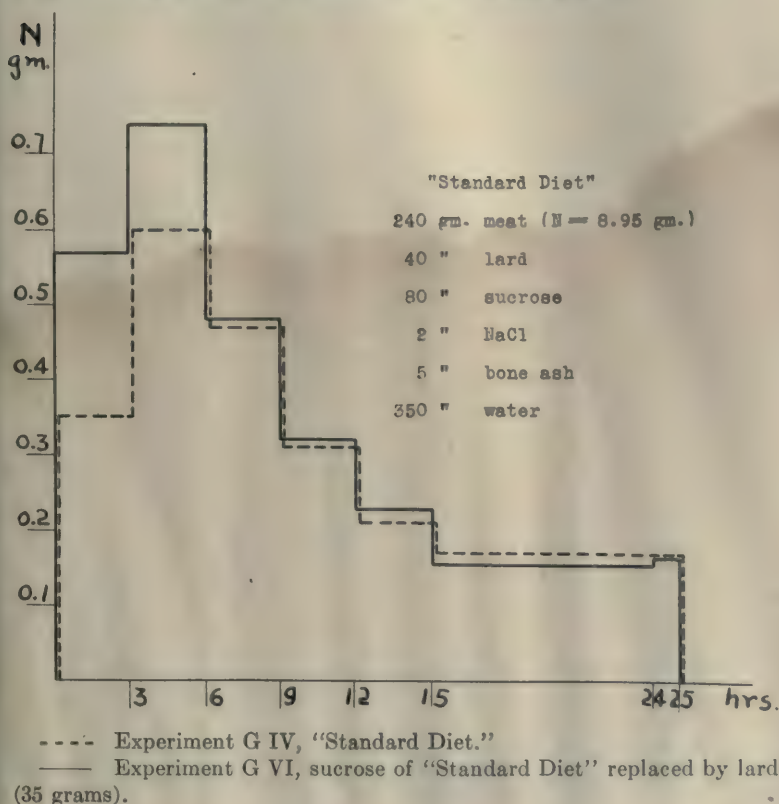
CURVE V. To illustrate the effect on the rate of nitrogen elimination of substituting *cotton-seed oil* for the non-nitrogenous constituents of the "Standard Diet."



Lard (Curve VI).

When lard was substituted for the sucrose of the "Standard Diet," the nitrogen excretion during the first two three-hour periods was considerably larger than in the "standard" experiment; afterwards the nitrogen-output curve was identical with that after the ingestion of the "Standard Diet."

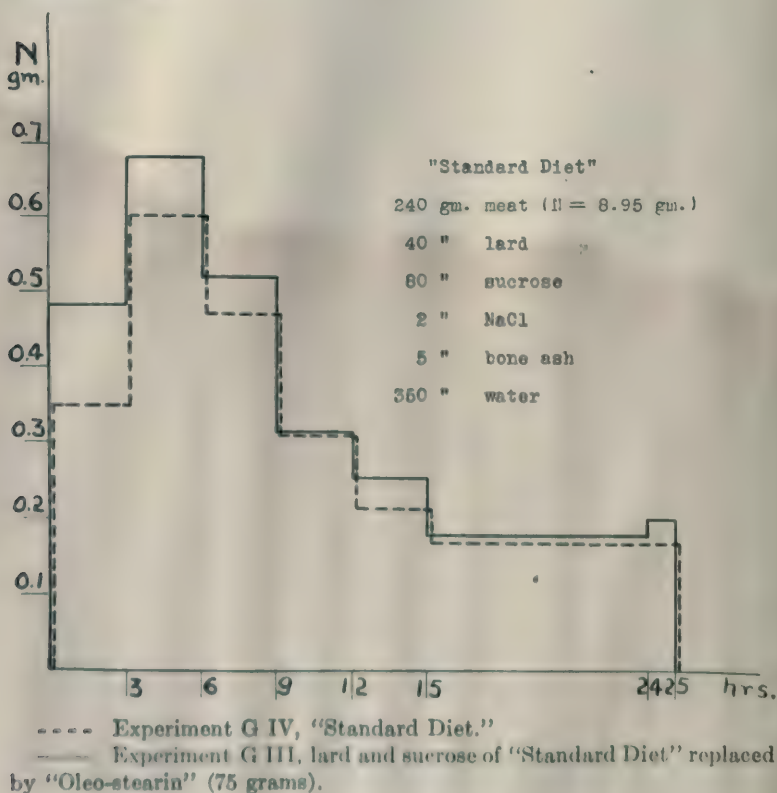
CURVE VI. To illustrate the effect on the rate of nitrogen elimination of substituting *lard* for the sucrose of the "Standard Diet."



"Oleo-stearin" (Substitution) (Curve VII).

The result of substituting "Oleo-stearin" for the non-nitrogenous parts of the "Standard Diet" was similar to that with lard, a nitrogen output above the normal occurring in the first three three-hour periods.

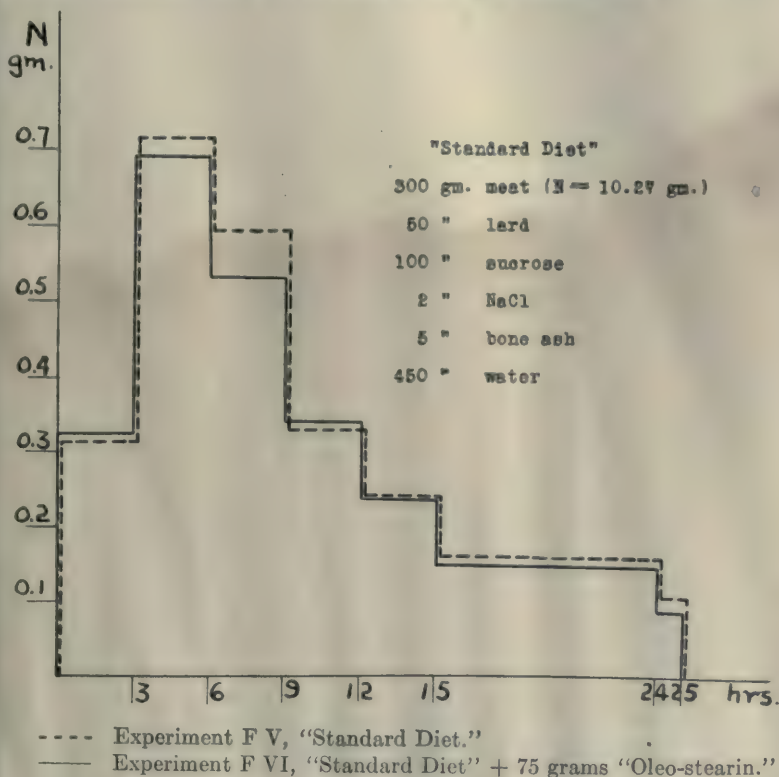
CURVE VII. To illustrate the effect on the rate of nitrogen elimination of substituting "Oleo-stearin" for the non-nitrogenous constituents of the "Standard Diet."



"Oleo-stearin" (Addition) (Curve VIII).

In the above experiments with fats the sugar was completely removed from the diet. When fat was *superimposed* on the "Standard Diet," the sucrose thus being retained, a nitrogen-output curve of the same character as that occurring after the ingestion of the "Standard Diet" followed.

CURVE VIII. To illustrate the effect of an *addition* of "*Oleo-stearin*" to the "Standard Diet" on the rate of nitrogen elimination.

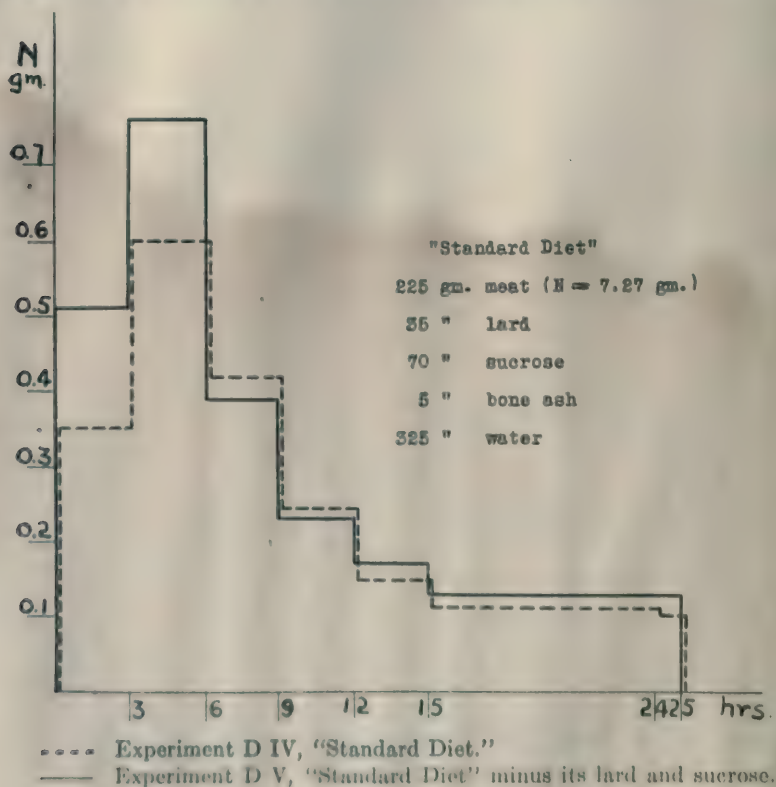


EXPERIMENT WITHOUT FAT OR CARBOHYDRATE IN THE DIET.

(Curve IX.)

In the discussion which follows reference will be made to an experiment where meat alone was given, the "Standard Diet" minus its content of sucrose and lard being fed. In the first two periods the nitrogen output under these conditions was larger than when the "Standard Diet" was fed intact; in the later periods, practically identical with the "standard."

CURVE IX. Comparison of the rate of nitrogen elimination after the ingestion of a mixed diet with that when meat alone is fed.



DISCUSSION.

The substitution of the different fats for the non-nitrogenous constituents of the "Standard Diet" did not yield concordant results. With cotton-seed oil the nitrogen-output curve was practically identical with the "standard;" with lard and "Oleo-stearin," higher in the first periods and afterwards the same as when the "Standard Diet" was fed. In these substitution experiments with fats two important changes in the diet were made: (a) a larger amount of fat was given; (b) carbohydrate was removed. One or both of these two changes must be responsible for the results obtained. The experimental data show that the typical "standard" curve was obtained when a large quantity of "Oleo-stearin" was *superimposed* on the "Standard Diet." Therefore, the larger amount of fat was not the important factor in causing the initial rise of the nitrogen-output curve above the normal when the non-nitrogenous constituents of the diet were replaced by "Oleo-stearin" (and *a priori* by lard). Furthermore, the nitrogen-output curve following the ingestion of the "Standard Diet" minus its content of sucrose and lard shows a close similarity to those obtained when lard and "Oleo-stearin," respectively, replaced the non-nitrogenous constituents of the "Standard Diet." In both types of experiment the sucrose of the "Standard Diet" has been removed. From the results obtained with carbohydrates⁸ it is evident that sucrose in the diet exerts a retarding influence on nitrogen excretion. It is not surprising, then, that with removal of the sucrose there was an increased output of nitrogen in the early periods. Obviously lard and "Oleo-stearin" *per se* have no influence on the rate of elimination of nitrogen.

With cotton-seed oil there was a slight preliminary delay in the nitrogen excretion. In the light of the foregoing discussion the effect of this fat must have been much more pronounced than is evident from the data. With the removal of the sucrose of the "Standard Diet" there would be a tendency for a more rapid elimination of nitrogen in the early periods. The fact that there is, on the contrary, a slower rate can only be explained by assuming that the cotton-seed oil has caused a marked retardation of the nitrogen excretion. This is made the more evident by contrasting

⁸ See the first part of this paper.

the nitrogen-output curve obtained when cotton-seed oil was present in the diet with that when meat alone was fed (cf. Curves V and IX).

The results with cotton-seed oil are in accord with those of earlier investigators who studied the influence of fat on the nitrogen-output curve. Panum (1874) and Feder (1881) found that lard caused a delay in the nitrogen excretion. Pari (1908) and Levene and Kober (1909) confirmed these findings; and Vogt (1906) likewise reported that fat (no mention of its character is made) caused a flattening of the nitrogen-output curve. All of these investigators used dogs as subjects of experimentation.⁹ The discrepancy between the results of the experiments with lard here reported and those of former workers awaits an explanation.

As causes of the retardation of the rate of nitrogen elimination on the part of cotton-seed oil there are several possibilities, viz: a delay in gastric discharge, a subnormal rate of absorption, altered metabolic conditions. The reports of Cannon¹⁰ that fat causes a retardation of gastric discharge, and of Vogt (1906) and Boettcher and Vogt (1909) that fat in the diet leads to a delayed absorption, make it quite probable that a sub-normal rate of alimentation is the prime cause of the results with cotton-seed oil; for no evidence has been found that altered metabolic conditions play a part in this connection.

The lack of concordance in the results obtained with the different fats may be explained by the fact that cotton-seed oil becomes more thoroughly incorporated in the diet and thus has a greater effect on the processes of alimentation, and hence on the nitrogen-output curve, than do the solid fats. An example of how varied the behavior of fats of different textures in the diet may be is afforded by experiments of Tangl and Erdélyi,¹¹ showing that the rate of discharge of fat from the stomach is dependent to a great extent upon its melting point and viscosity.

⁹ Although the results have no bearing on the experiments here reported, it should be noted that Wolf (1912) studied the effect on the nitrogen-output curve of feeding fat to a fasting man.

¹⁰ Cannon: *Amer. Journ. of Physiol.*, xii, p. 387, 1904.

¹¹ Tangl and Erdélyi: *Biochem. Zeitschr.*, xxxiv, p. 94, 1911.

SUMMARY OF RESULTS WITH FATS.

The effect on the nitrogen-output curve of replacing the non-nitrogenous constituents of a mixed diet by fat varied with the character of the fat as follows: (a) with the fluid cotton-seed oil there was a slower rate of nitrogen elimination; (b) with lard and "Oleo-stearin" the nitrogen excretion in the early periods following the meal was above the normal. The apparent action of these latter fats was shown to be in reality the result of removing the sucrose from the diet.

The action of cotton-seed oil *per se* was to cause a marked delay in the rate of elimination of nitrogen. Neither lard nor "Oleo-stearin" by themselves had any effect on the nitrogen-output curve.

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THE RATE OF ELIMINATION OF NITROGEN AS INFLUENCED BY DIET FACTORS.

III. THE INFLUENCE OF THE CHARACTER OF THE INGESTED PROTEIN.

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(Received for publication, August 4, 1913.)

The influence which the character of the protein intake has on the rate of nitrogen elimination may be considered to better advantage now that the effect of various other diet factors has been determined.¹ The fact that there has been considerable discussion in recent years as to whether or not all proteins are catabolized with equal rapidity gives stimulus to further research on this subject.

In comparison with previous experiments the "Standard Diet" used in the present study contained a small amount of water;² and the relative amounts of fat and carbohydrate were changed.³ With this low water intake two of the animals invariably gave an atypical "standard" curve of nitrogen output, the maximum excretion not occurring until the third three-hour period instead of in the second.⁴ The fact that there was a delay in nitrogen excretion when these animals were given the modified "Standard

¹ Cf. Mendel and Lewis: this *Journal*, xvi, pp. 19 and 37, 1913.

² The proteins were all in the form of dry powders and, if an amount of water as large as had been given were now used, it was thought that there would be difficulty in getting the animal to eat the entire ration.

³ Inasmuch as carbohydrates have been shown to exert a retarding influence on the rate of nitrogen elimination (cf. Mendel and Lewis: this *Journal*, xvi, p. 37, 1913), it seemed advisable to reduce the amount of carbohydrate in the diet. Except for the changes in the "Standard Diet" we employed the methods of our first paper (this *Journal*, xvi, p. 19, 1913).

⁴ Cf. Mendel and Lewis: this *Journal*, xvi, p. 23, 1913.

Diet" could not be explained by the change in the relative amounts of fat and sugar in this diet.⁵ The only other explanation, then, was that the variation was due to the decrease in the water intake. This possibility was suggested by the comparatively low volume of urine per kilo of body weight of these atypical animals. In order to determine whether this hypothesis was correct, an experiment was conducted on one of these animals in which the water content of the diet was made proportional to that of meat.⁶ This time the typical "standard" curve with the maximum output of nitrogen in the second three-hour period was obtained. Thus it is conclusive that too great a diminution in the intake of water will cause a marked slowing of the rate of elimination of nitrogen in the urine. In the experiments to be reported the results are in all cases compared with those obtained on the same animal after the ingestion of this modified diet, a fact which the reader should bear in mind.

PRELIMINARY EXPERIMENTS.

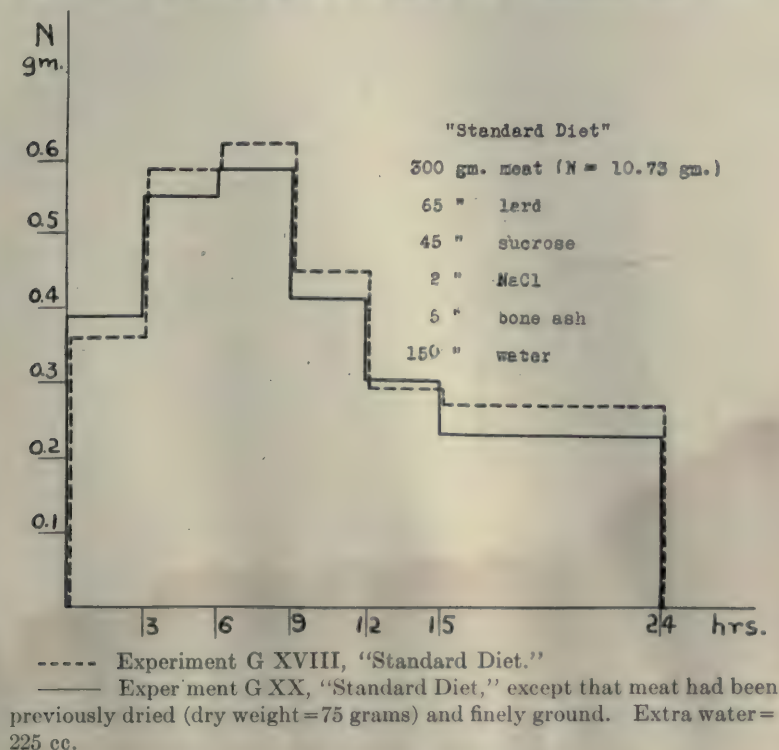
Dried meat (Curve I).

In the present study the rate of elimination of nitrogen was determined after the ingestion of several different proteins, the majority of which were in the form of dry powders. The difference in texture between such dry preparations and moist meat suggested itself as a possible cause for a variation in the nitrogen-output curve and so demanded a preliminary investigation. For this purpose a day's portion of meat was dried at about 55°C., then finely ground in a coffee mill, and fed. The drying of meat had practically no effect on the rate of nitrogen elimination.

⁵ In a previous paper (this *Journal*, xvi, p. 37, 1913) the authors have shown that carbohydrate in the diet causes a delay in nitrogen excretion. The reduction of the amount of carbohydrate in this case would tend to have an opposite effect.

⁶ The water addition in our original "Standard Diet" was calculated on this basis (cf. this *Journal*, xvi, p. 19, 1913).

CURVE I. To illustrate the absence of effect on the rate of elimination of nitrogen of previously drying the meat of the "Standard Diet."

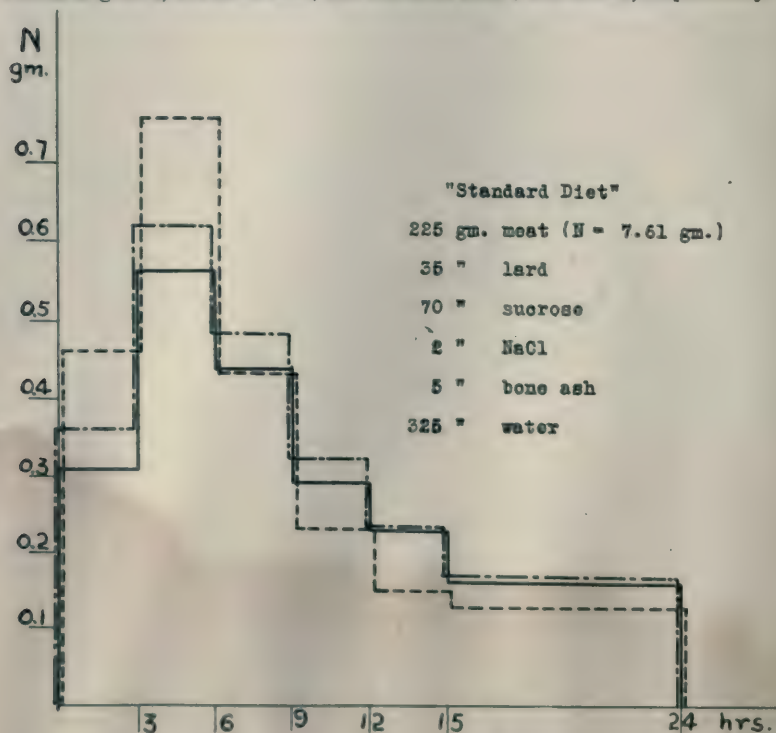


*Extracted meat*⁷ (Curve II).

Inasmuch as a notable proportion of the nitrogen of meat is non-protein in character, it seemed desirable to study meat devoid of extractives and in such a form as to be directly comparable with the extractive-free isolated proteins. The nitrogen-output curves of the latter are compared with that occurring after the ingestion of an extracted meat powder. The use of such an extractive-free meat is made the more necessary because of the recognized influence of extractives on secretory processes in the stomach, and because the nitrogen of extractives like creatine and the purines is in a different chemical structure from that

⁷ A light brown, impalpable powder obtained from Armour and Company.

CURVE II. Comparison of the rates of nitrogen elimination on diets containing *meat*, *extracted meat*, and *extracted meat+extractives*, respectively.



----- Experiment D XXIV, "Standard Diet."

———— Experiment D XXX, meat of "Standard Diet" replaced by 57 grams extracted meat (N=7.65 grams) and 170 grams water.

- - - Experiment D XXXI, meat of "Standard Diet" replaced by 51 grams extracted meat (N=6.84 grams), beef extract (N=0.77 grams) and 170 grams water.

of the familiar amino-acids. The nitrogen-output curve with the extracted meat was considerably more flattened than that after the ingestion of fresh meat—a fact which may be explained in part by the absence of extractives in the former product, the following experiments showing that extractive-nitrogen is very rapidly eliminated.

Extracted meat + Extractives (Curve II).

When extracted meat furnished 90 per cent, and Liebig's beef extract 10 per cent of the nitrogen of the diet, there was a larger nitrogen output in the first periods than after the ingestion of extracted meat alone, showing that extractive-nitrogen is eliminated with comparative rapidity.

Meat + Urea (Curve III).

When the nitrogen of the diet was furnished in equal portions by meat and urea, the nitrogen excretion in the first two periods was enormously larger than when meat alone was fed. It is evident that the urea-nitrogen is rapidly eliminated.⁸

In the first of these two experiments (Curve II) extractive-nitrogen was present in approximately the same proportion as it occurs in meat; the nitrogen-output curve, however, was considerably more flattened than when fresh meat was fed. It is quite apparent, therefore, that the absence of extractives can only account in part for the slower rate of nitrogen elimination when extracted meat replaces the meat of the "Standard Diet." The cause of this difference between the nitrogen-output curve of fresh meat and that of extracted meat may be that the latter product is richer in connective tissue and so less readily digestible. The finding of Mendel and Fine⁹ that this same extracted meat was not as well utilized as fresh meat bears out such an assumption.

PROTEIN MATERIALS EMPLOYED BESIDES MEAT.

In several cases the materials used in the present study were isolated proteins; in others, products relatively rich in protein. A description of each of the substances used follows.

Casein¹⁰—a purified preparation in the form of an impalpable powder containing 13.36 per cent nitrogen.

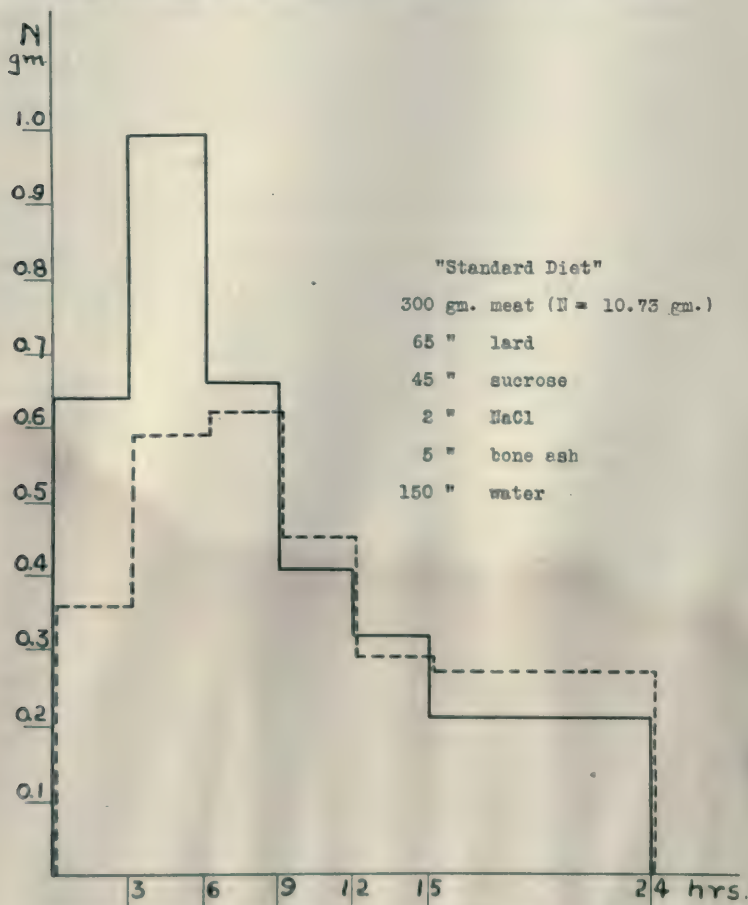
⁸ Wolf (1912b) and Cathcart and Green (1913) have reported a rapid elimination of nitrogen after feeding urea to man.

⁹ Mendel and Fine: this *Journal*, xi, p. 5, 1912.

¹⁰ The casein and a portion of the edestin were contributed by Dr. T. B. Osborne, New Haven, Conn.

Rate of Nitrogen Elimination

CURVE III. To illustrate the effect on the rate of elimination of nitrogen of replacing one-half of the meat of the "Standard Diet" by urea.



Ovovitellin¹¹—a purified product; an impalpable powder containing 13.78 per cent nitrogen.

Edestin—a purified preparation. Two lots of this material were used, both of which were impalpable powders containing 16.23 per cent and 16.95 per cent of nitrogen, respectively.

“Glidine”¹²—a commercial preparation from wheat;¹³ an impalpable powder giving no starch reaction and containing 14.8 per cent nitrogen.

Gelatin—a commercial preparation in the form of a fine powder containing 15.1 per cent nitrogen.

Soy Bean¹⁴—an impalpable powder containing 7.25 per cent nitrogen, thus being poor in protein as compared with the other dry materials used. Besides protein the soy bean contains a large amount of fat and considerable cane sugar.¹⁵

Liquid Egg-White—the whites of eggs thoroughly strained and mixed (nitrogen content=1.95 per cent).

Dried Egg-White—the whites of eggs dried at 50°C. and then ground to a fine powder in a mortar (nitrogen content=13.6 per cent).

Coagulated Egg-White—the whites of hard boiled eggs passed through a sieve (nitrogen content=1.92 per cent).

Ovalbumin¹⁶—a purified product in the form of an impalpable powder containing 15.4 per cent nitrogen.

Water was added to the powdered proteins (with the exception of gelatin, dried egg-white, and ovalbumin) the night previous to feeding, for the purpose of allowing ample time for “hydration” of the material. The following morning a thoroughly hydrated mush was always found.

¹¹ Prepared by Mr. R. L. Kahn in our laboratory.

¹² Obtained from Menley and James, New York City.

¹³ This material, “according to Bergell, and Thiemar, is prepared from wheat flour by a process of washing and centrifuging.”

¹⁴ Mr. M. F. Deming of the Cereo Company, Tappan, New York, contributed this material.

¹⁵ For a complete analysis of soy bean, see Ruhräh: *Journ. Amer. Med. Assn.*, liv, p. 1664, 1910; also quoted by Mendel and Fine: *this Journal*, x, p. 435, 1911.

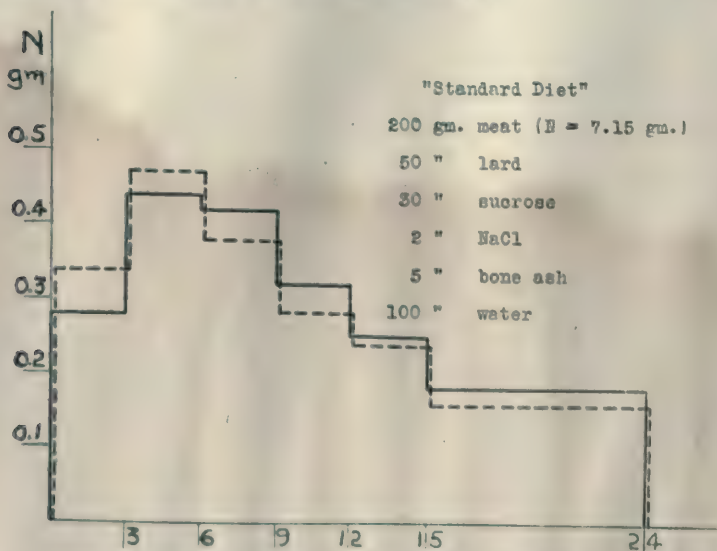
¹⁶ This material was prepared in our laboratory by Dr. Martha Tracy.

EXPERIMENTS WITH PROTEINS.

Casein (Curve IV).

When casein was substituted for the meat of the "Standard Diet," the nitrogen-output curve was practically the same as that with extracted meat.

CURVE IV. Comparison of the rates of nitrogen elimination on diets containing *extracted meat* and *casein*, respectively.



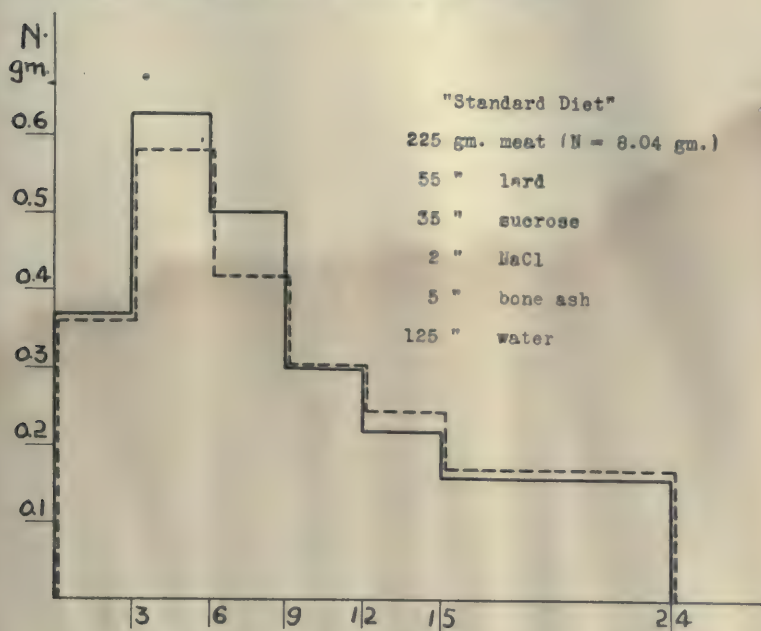
----- Experiment II VII, meat of "Standard Diet" replaced by 53 grams extracted meat (N=7.11 grams) and 150 grams water.

———— Experiment II VIII, meat of "Standard Diet" replaced by 54 grams casein (N=7.21 grams) and 150 grams water.

Ovovitellin (Curve V).

The rate of nitrogen elimination after the ingestion of ovovitellin was identical within the limits of experimental error with that when extracted meat was fed.

CURVE V. Comparison of the rates of nitrogen elimination on diets containing *extracted meat* and *ovovitellin*, respectively.



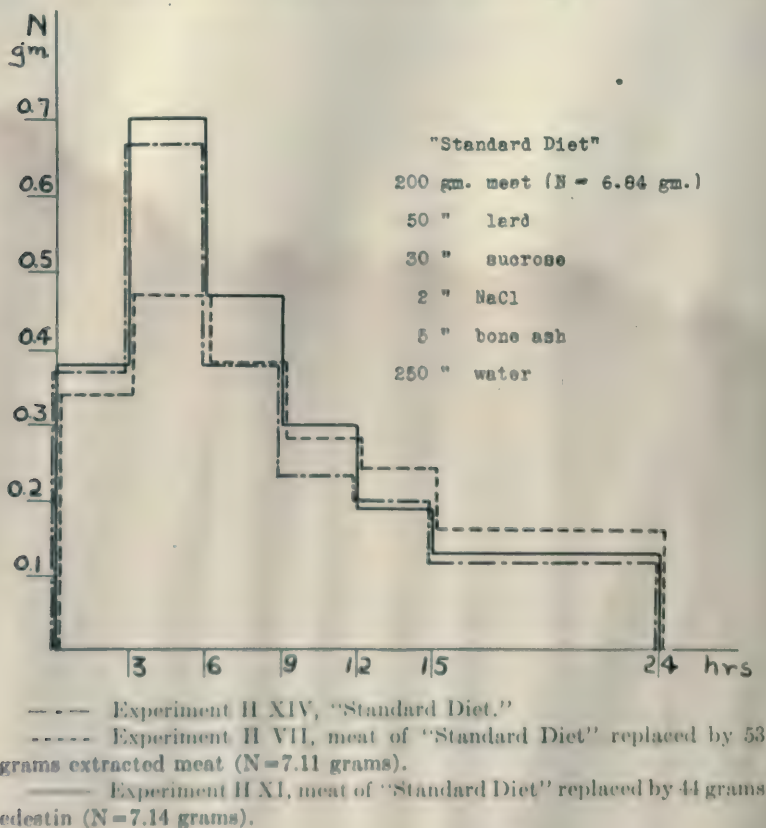
----- Experiment D XXXIII, meat of "Standard Diet" replaced by 60 grams extracted meat (N=8.05 grams) and 170 grams water.

———— Experiment D XXXVIII, meat of "Standard Diet" replaced by 58 grams ovovitellin (N=7.99 grams) and 170 grams water.

Edestin (Curve VI).

The nitrogen-output curve with edestin was not of such a flattened aspect as that with extracted meat. The nitrogen excretion in the earlier periods was larger; in the later periods, smaller than with extracted meat. The edestin curve, however, was very much the same as that with fresh meat.

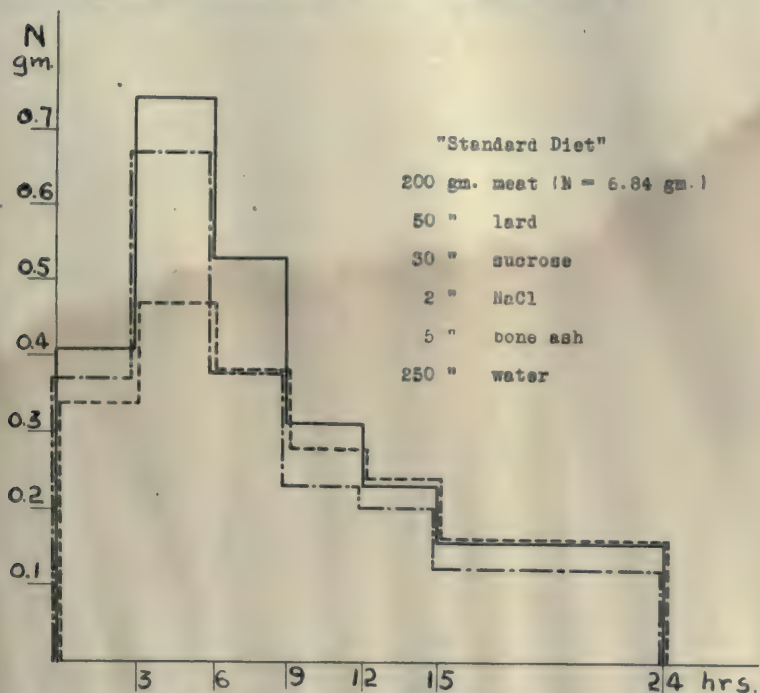
CURVE VI. Comparison of the rates of nitrogen elimination on diets containing *meat*, *extracted meat*, and *edestin*, respectively.



"Glidine" (Curve VII).'

When "Glidine" constituted the protein intake, the nitrogen excretion was larger during the earlier periods of the day than with extracted meat. The character of the nitrogen-output curve was practically the same as that with fresh meat; the two curves ran parallel, that with "Glidine" being at a higher level.

CURVE VII. Comparison of the rates of nitrogen elimination on diets containing meat, extracted meat, and "Glidine," respectively.

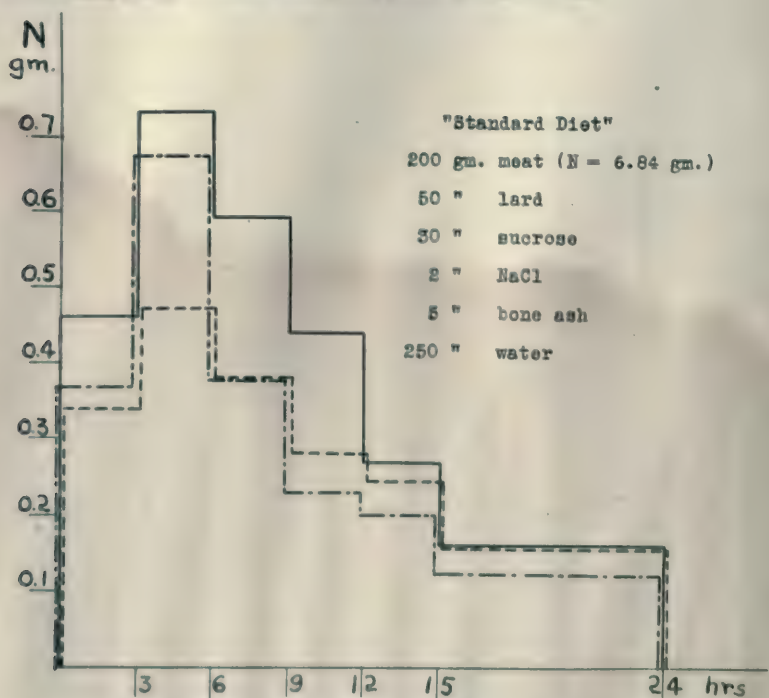


- Experiment H XIV, "Standard Diet."
- Experiment H VII, meat of "Standard Diet" replaced by 53 grams extracted meat (N=7.11 grams).
- Experiment H IX, meat of "Standard Diet" replaced by 48 grams "Glidine" (N=7.10 grams).

Gelatin (Curve VIII).

Again with gelatin the nitrogen-output curve was not as flattened as that with extracted meat, but identical in character with the "standard" (fresh meat) curve. There was a negative balance with gelatin and the nitrogen excretion for all the periods was higher than with meat.

CURVE VIII. Comparison of the rates of nitrogen elimination on diets containing *meat*, *extracted meat*, and *gelatin*, respectively.

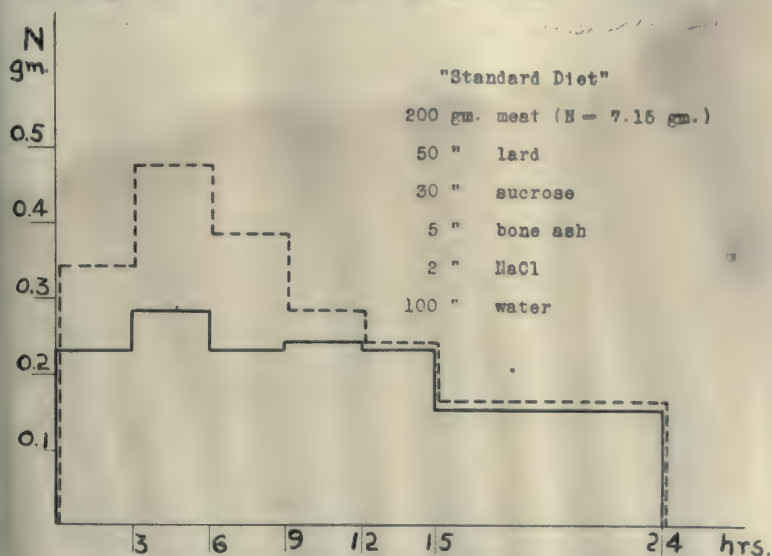


- Experiment H XIV, "Standard Diet."
 ---- Experiment H VII, meat of "Standard Diet" replaced by 53 grams extracted meat (N=7.11 grams).
 -.-.- Experiment H X, meat of "Standard Diet" replaced by 47 grams gelatin (N=7.08 grams).

Soy bean (Curve IX).

On account of the comparatively poor utilization of soy bean¹⁷ its ingestion was followed by a smaller nitrogen output in all the periods of the day than when extracted meat was fed. Furthermore, this lower curve was not parallel to that with extracted meat; its character was quite different. In the earlier periods of the experiment here reported a smaller percentage of the 24-hour nitrogen output was excreted than when meat was fed; in the later periods, a larger percentage. In a second experiment the maximum nitrogen excretion did not occur until the third three-hour period, instead of the second. In both cases, then, there was a delay in the elimination of nitrogen independent of the poorer utilization of the soy bean.

CURVE IX. Comparison of the rates of nitrogen elimination on diets containing *extracted meat* and *soy bean*, respectively.



----- Experiment H VII, meat of "Standard Diet" replaced by 53 grams extracted meat (N=7.11 grams) and 150 grams water.

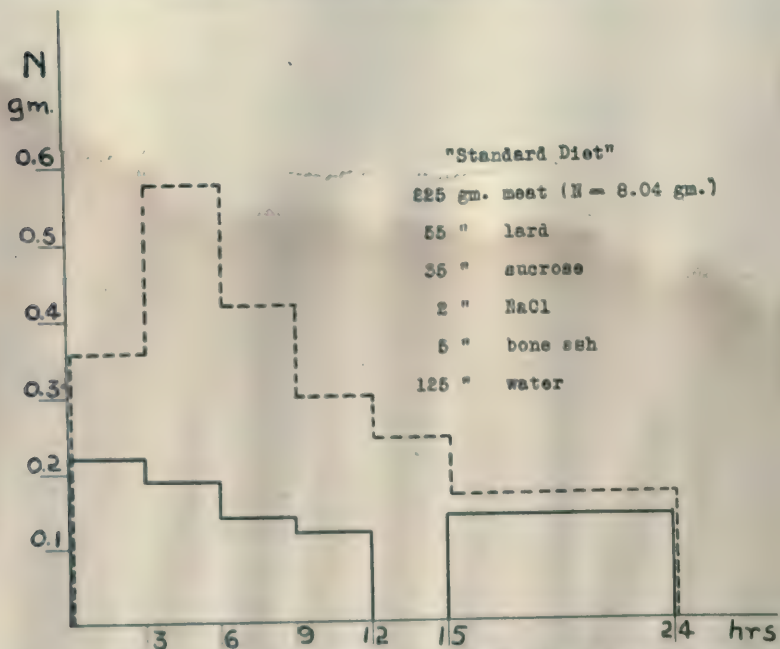
———— Experiment H XII, meat of "Standard Diet" replaced by 99 grams soy bean (N=7.18 grams) and 150 grams water.

¹⁷ Mendel and Fine (this *Journal*, x, p. 345, 1911) found soy bean nitrogen to be poorly utilized.

Uncoagulated egg-white (Curve X).

On the days when native egg-white was fed the nitrogen output was only about half as large as the intake. It is probable, as the discussion to follow will show, that this material was poorly utilized. The character of the nitrogen-output curve with uncoagulated egg-white was somewhat different from that with extracted meat, the maximum excretion occurring during an earlier period—in two experiments during the first three-hour period,

CURVE X. Comparison of the rates of nitrogen elimination on diets containing *extracted meat* and *liquid egg-white*, respectively.



----- Experiment D XXXIII, meat of "Standard Diet" replaced by 60 grams extracted meat (N = 8.05 grams) and 170 grams water.

———— Experiment D XXXII, meat and water of "Standard Diet" replaced by 412 cc. liquid egg-white (N = 8.03 grams).¹²

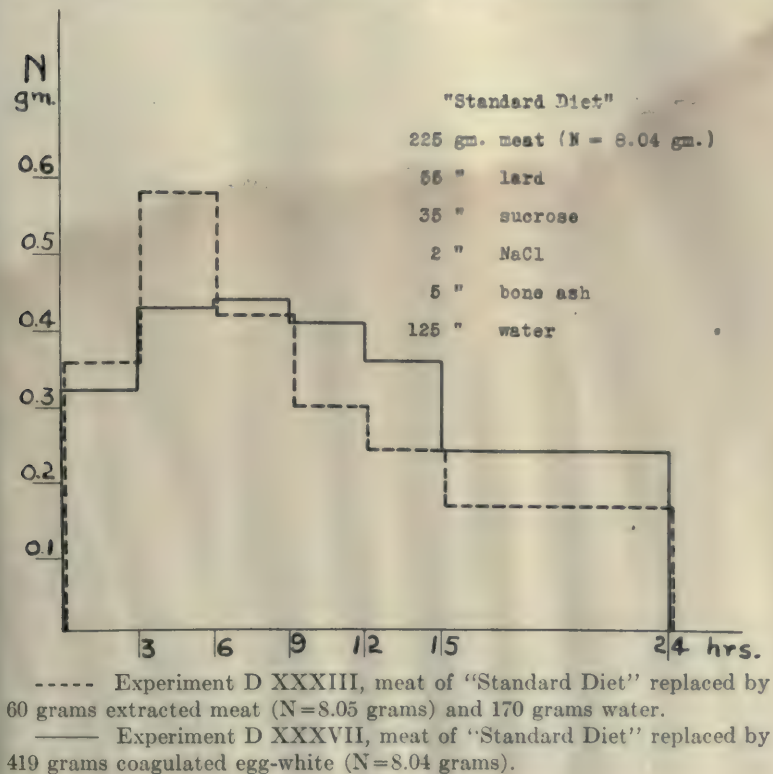
¹² Diarrhea during 5th three-hour period; urine contaminated. The ingestion of dried native egg-white was also followed by diarrhea.

instead of during the second; in a third experiment during the second period, instead of during the third. The results with liquid egg-white and dried egg-white, respectively, were concordant.

Coagulated egg-white (Curve XI).

Coagulated egg-white was evidently well utilized; but the nitrogen-output curve after its ingestion was more flattened than that when extracted meat was fed. In other words there was a comparative delay in the elimination of nitrogen when coagulated egg-white constituted the protein intake.

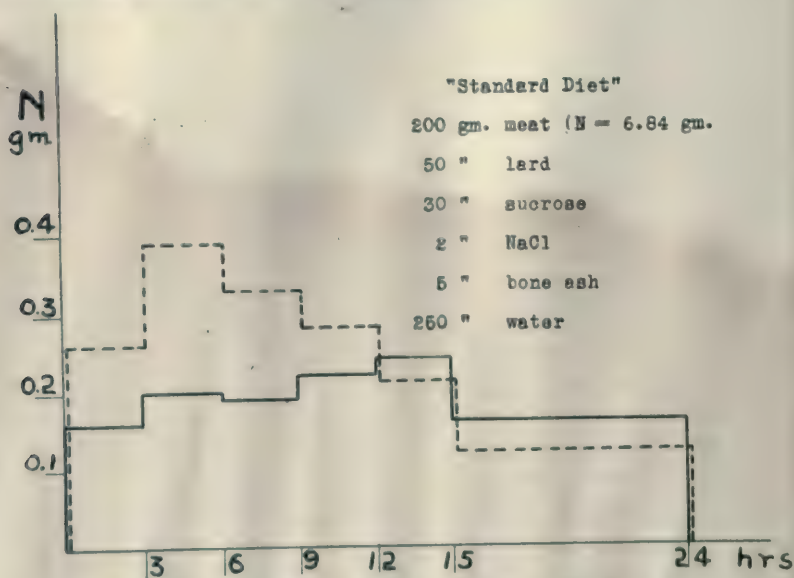
CURVE XI. Comparison of the rates of nitrogen elimination on diets containing *extracted meat* and *coagulated egg-white*, respectively.



*Ovalbumin*¹⁹ (Curve XII).

When ovalbumin was fed the urinary nitrogen-output was much smaller than the intake, the marked diarrhea about nine hours after the meal suggesting a poor utilization of the ovalbumin as the cause of the smaller nitrogen excretion. Furthermore, the rate of nitrogen elimination with this material was quite different from that with extracted meat. There was a rise to a maximum in the second three-hour period, then a slight fall during the third three hours, followed by a second rise to a maximum in the fifth

CURVE XII. Comparison of the rates of nitrogen elimination on diets containing *extracted meat* and *ovalbumin*, respectively.



----- Experiment H XV, meat of "Standard Diet" replaced by 51 grams extracted meat (N=6.84 grams).

———— Experiment H XVII, meat of "Standard Diet" replaced by 50 grams ovalbumin (N=6.73 grams).²⁰

¹⁹ We are greatly indebted to Mr. R. L. Kahn for performing this and several other experiments of the present series.

²⁰ Voluminous diarrhea during third three-hour period; animal ate some feces.

period. The fact that the animal ate a portion of the diarrheal feces at the beginning of the third period may account in part for the second rise.

DISCUSSION.

The nitrogen-output curves following the ingestion of unaltered meat and extracted meat powder, respectively, were quite different. The slower rate of elimination of nitrogen when extracted meat was fed cannot be explained by the dry condition of this material; for the nitrogen-output curve was unchanged by a previous drying of the meat of the "Standard Diet." The absence of extractives in the extracted meat can only account in part for the delayed nitrogen excretion. This product presumably contains proportionately more connective tissue than the fresh meat used and thus is digested more slowly. The nitrogen-output curves following the ingestion of casein and ovovitellin are practically identical with that of extracted meat; the character of the curves with edestin, "Glidine," and gelatin is the same as that with fresh meat.

Soy bean, egg-white, and isolated ovalbumin gave nitrogen-output curves radically different from those of either of the meat products studied. The comparative delay in nitrogen elimination independent of the poor utilization of soy bean may be explained in part by a greater difficulty of digestion of this product, and in part by the presence of sucrose in soy bean, it having been shown in a previous paper²¹ that the presence of carbohydrate in the diet delays nitrogen excretion. The comparatively small excretion of nitrogen after the ingestion of native egg-white and ovalbumin is caused in all probability by a poor utilization of these materials, the early diarrhea following their ingestion making such an explanation quite likely. That uncoagulated ovalbumin is poorly utilized was reported by Falta (1906), who found that the coefficient of utilization of this material in man was only about 70 per cent. Wolf (1912b) fed a large quantity of native egg-white to man and reported that only about half was utilized. When liquid egg-white²² was fed it is probable that very little gastric proteolysis occurred;

²¹ Mendel and Lewis: this *Journal*, xvi, p. 37, 1913.

²² The dried egg-white mixed with water would be essentially the same as the natural product.

for Cannon,²¹ and London and Sulima,²² working with cats and dogs, respectively, have reported that this material begins to leave the stomach almost immediately after ingestion. The early discharge of the stomach, the comparatively early emptying of the bowel, and the unfavorable character of liquid egg-white for the action of digestive enzymes, together with a possible resistance of native protein to digestion, may all contribute to a poor utilization of this material. Coagulated egg-white was well utilized; but following its ingestion there was a comparative delay in nitrogen excretion. The slower rate of elimination of nitrogen with this source of protein cannot be accounted for by a delay of gastric discharge; for Cannon, and London and Sulima have demonstrated that egg-white coagulated by heat leaves the stomach more rapidly than most proteins. It is probable that the delayed excretion of nitrogen may be caused in part, at least, by a comparative difficulty of digestion of coagulated egg-white on account of the compact and impermeable character of the fine particles of coagulum.

A few reports in the literature are in harmony with this view that such changes as do occur in the rate of elimination of nitrogen after the ingestion of different protein materials may be explained by variations in alimentary, rather than metabolic processes. Van Slyke and White (1911), using the method of the present work, demonstrated that different nitrogen-output curves were obtained after feeding various boiled fish meats to a dog; and attributed this result to a variation in the rate of digestion. The validity of such an explanation is made very clear by a comparison of the results obtained by these authors with fresh and salt cod, there being as much difference in the nitrogen-output curves of the two preparations of this one fish as in those of any of the different fish. Vogt (1906) investigated the effect on the rate of elimination of nitrogen of superimposing various proteins in considerable quantities on a standard diet, finding that both coagulated and uncoagulated ovalbumin caused a delay in nitrogen excretion whereas edestin and a casein preparation (Nutrose) gave a nitrogen-output curve of approximately the same character

²¹ Cannon: *Amer. Journ. of Physiol.*, xii, p. 387, 1904.

²² London and Sulima: *Zeitschr. f. physiol. Chem.*, xlvi, p. 232, 1905.

as meat. This author believed that the delay with ovalbumin was caused by a comparatively slow rate of digestion of this material. Loeb (1911) studied the rate of elimination of nitrogen after replacing about one-half of the meat of a standard diet by another form of protein; and demonstrated that there was only a very slight change when meat and casein, respectively, were fed, although considerable difference existed between the curves of these proteins on the one hand and those of their hydrolyzed products on the other. In experiments where the urine was collected only for twelve-hour periods Falta, Grote, and Staehelin (1907) found approximately the same rate of nitrogen excretion with casein as with meat. All of these investigators worked on dogs. Wolf (1912a, 1912b) added various proteins and non-proteins to a "standard" diet in man and collected the urine in hourly periods, studying among other things the rate of elimination of nitrogen. He found little difference in the nitrogen-output curves following the ingestion of gelatin and plasmon, respectively. With veal, however, there was a somewhat slower rate of nitrogen output. Native egg-white and coagulated egg-white gave results quite similar to those reported in the present paper. Wolf (1912c) also studied the rate of nitrogen elimination in dogs after feeding cooked and raw meat, respectively, obtaining practically identical results in the two cases.

In considering the significance of the results of the present study attention should be given to Falta's conclusions from his work on the rate of metabolism of proteins. The method of investigation employed by this author was to determine the average daily nitrogen-output of a dog in nitrogen equilibrium for a period of several days, then to superimpose the protein to be studied on the "standard" diet, and to ascertain how long a time was required for a reappearance of an excess of nitrogen in the urine equivalent to the nitrogen of the superimposed material. Falta (1904 and 1906) studied different proteins on man and found that with most of these more than half of the excess nitrogen reappeared on the first day, about three days being required for the entire amount to show up. With casein, for example, about two-thirds of the excess nitrogen reappeared during the first day, and most of the remainder on the second day. A few exceptions occurred, however,

the most striking being with ovalbumin and ovovitellin.²³ In these cases only about 27 per cent of the excess nitrogen appeared the first day; and five days were required for all to reappear, although all but a very small amount had come out in three days. When coagulated ovalbumin was the added protein no longer time was required for the reappearance of the excess nitrogen than was the case with casein. With dogs the results with ovalbumin and casein were the same as for casein with man. These observations on man were confirmed by Hämäläinen and Helme (1907), who demonstrated that a longer time was required for the reappearance of the excess nitrogen with egg-white than with a casein preparation (Proton) or roast veal. Cathcart and Green (1913), employing the Falta method of superimposition on man, reported that with egg-white, both coagulated and uncoagulated, only a small part of the extra nitrogen appeared in the urine even after several days. There was little difference in the rate of elimination of the extra nitrogen after adding veal and gelatin, respectively, to the diet, greater differences being obtained with the same sample of gelatin in two experiments where the basal rations varied considerably. Vogt (1906) used Falta's method of study on dogs and found that a longer time was required for the reappearance of the excess nitrogen when egg-white, both uncoagulated and coagulated, was superimposed on the standard diet than when edestin or a casein preparation, Nutrose, was added. All of these communications are in harmony with that of Graffenberger (1891), who showed by a somewhat different method that when gelatin or fibrin was superimposed on a standard diet the excess nitrogen reappeared more rapidly than when peptone constituted the increased nitrogen intake.

From the results of his experiments Falta (1906) has concluded that the longer time required for the reappearance of the excess nitrogen after superimposing ovalbumin on the standard diet was the result of the absorption of comparatively large cleavage products of this protein, a longer time being required for the catabolism of these higher protein residues. Hämäläinen and Helme (1907) held a similar view; and Levene (1909a, 1909b, 1909c, 1910) and his co-workers from a series of studies of an entirely different

²³ Only one experiment with ovovitellin is reported and the author says that no definite conclusion should be drawn from a single experiment.

nature likewise came to the conclusion that the higher protein cleavage products are catabolized more slowly than the simple amino-acids. Vogt (1906) was not inclined to favor such an explanation, maintaining that a slower rate of digestion and absorption might account in part for the results obtained by him with egg-white, and that certain unknown factors of intermediary metabolism might play a part.

Although Falta's experiments are not directly comparable with those of the present study, yet if one recalls how readily texture of the diet influences the rate of digestion and absorption independently of the character of the protein, it seems quite likely that Falta's results were caused in part by a difference in the rate of digestion of the various materials studied. Let us consider what would be the effect of a markedly delayed digestion and absorption in studies of the type that Falta made. In the experiments of this author on man the superimposed protein as well as the standard diet was fed in four portions distributed over the day. Under such conditions it is quite likely that absorption of the digestion products of a difficultly digestible protein would not be complete until after the beginning of the second day. It is not surprising, then, that the excess nitrogen eliminated on the day when the protein was added to the diet should be smaller than when the superimposed protein was readily digestible; nor that it should be greater on the following day, the amount of nitrogen absorbed on this second day being greater than that usual on a normal day. The fact that, when Falta fed a single meal to dogs at the beginning of the day, he obtained a result with ovalbumin similar to that with casein makes such an explanation more probable; for in this case digestion and absorption would certainly be complete during the first day.

SUMMARY OF RESULTS WITH PROTEINS.

The nitrogen-output curves following the ingestion of meat and extracted meat, respectively, differ considerably, that with the latter product being more flattened. This slower rate of elimination of nitrogen cannot be explained by the dried condition of the extracted meat; and only in part by the absence of extractives in this material. It is suggested that the extracted meat may

have contained proportionately more connective tissue than the fresh meat used and thus have been less readily digestible.

The nitrogen-output curves following the ingestion of most of the proteins studied—casein, ovovitellin, edestin, "Glidine," gelatin—differ in character to no greater extent than those obtained by feeding the two meat products employed. With egg-white, ovalbumin, and soy bean, however, curves of a character radically different from that of either of the meats were obtained. These results may be explained to a great extent by a difference in the rate and completeness of digestion and absorption of these materials; while the sucrose in the soy bean will also account in part for the delay in nitrogen elimination with this product. When these alimentary differences are duly taken into account, the conclusion seems justified that proteins do not differ materially in their rate of metabolism.

Falta's conclusions from his work on the rate of protein metabolism and the similar conclusions of Hämäläinen and Helme, and of Levene and his co-workers were discussed. From the results of the present study it seems quite probable that the findings of these authors may be explained by other factors than an assumed difference in the rate of metabolism of proteins caused by an absorption of larger or smaller cleavage products.

The results of the experiments reported in the papers of this series show that apart from the character of the protein ingested a large number of diet factors—the water intake, the presence and nature of indigestible materials in the diet, the amount and character of the carbohydrate fed, and to some extent the presence of fat in the diet—play a rôle in modifying the rate of elimination of nitrogen after a meal containing protein. With most of the proteins studied the nitrogen-output curves differed to only a slight extent from one another; and in no case did the nature of the protein have a greater effect on the rate of nitrogen elimination than some of the non-protein diet factors mentioned above.

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THE CARBON DIOXIDE AND OXYGEN CONTENT OF THE BLOOD AFTER CLAMPING THE ABDOMINAL AORTA AND INFERIOR VENA CAVA BELOW THE DIAPHRAGM.

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In search of experimental support for the over-production theory of diabetes mellitus Porges¹ and Porges and Salomon² have found that ligation of the abdominal aorta and inferior vena cava just below the diaphragm, both in normal rabbits and in depancreatized dogs, causes a rise in the external³ respiratory quotient. They interpret this result as proof (1) that the organism is dependent upon the liver for its power to oxidize protein and fat, carbohydrate only or carbohydrate chiefly being oxidized when the liver is excluded; and (2) that the depancreatized animal retains its power to oxidize sugar.

A rise in respiratory quotient after this radical interference with the circulation has been confirmed by Rolly,⁴ who, however, finds the rise not at all constant and gives an altogether different explanation. Verzář⁵ likewise witnessed a sudden change in the R. Q., but a change in the same direction, when the liver was partially excluded by anastomosis of the portal vein with the lower

¹ Porges: *Biochem. Zeitschr.*, xxvii, p. 131, 1910.

² Porges and Salomon: *Ibid*, p. 143.

³ The term *external* R.Q. is used here in order to emphasize the fact that the exchange of gases between blood and outside air does not under all circumstances take place at the same rate as the exchange between blood and tissue. The volumetric relations between the CO₂ gain of venous blood and O₂ loss from arterial may be called the *internal* R.Q.

⁴ Rolly: *Deutsch. Arch. f. klin. Med.*, cv, p. 494, 1912; *Münch. med. Wochenschr.*, 1912, Nos. 22 and 23.

⁵ Verzář: *Biochem. Zeitschr.*, xxxiv, p. 52, 1912.

part of the inferior vena cava (Queirolo operation). Fischler and Grafe⁶ ligated the hepatic artery of dogs which, some weeks before, had been successfully operated for the Eck fistula, and found in two out of six cases a distinct rise in the R. Q. Böhm⁷ reports but a very slight rise "after exclusion of the abdominal organs even in depancreatized dogs."

In all these experiments showing a higher R. Q.⁸ there is, as would be expected, a reduction in the total respiratory exchange, depending in amount upon the kind and amount of tissue excluded from the circulation. The reduction in the absorption of oxygen is greater than that for the elimination of carbon dioxide. Hence the higher R. Q. In other words, after the crucial operation there is, relatively, a greater output of CO₂.

There are the following possible ways of explaining this result: (1) A greater production of CO₂ with no change in the rate of elimination. If carbohydrate or carbohydrate-like bodies should be oxidized instead of protein or fat, more CO₂ would be produced. This is the explanation adopted by the von Noorden school. (2) Greater elimination of CO₂ from the blood with no essential change in the rate of production. The influences which may be conceived of as driving out more CO₂ may be (a) chemical or (b) mechanical. If more acids were produced, or if acids produced as usual were not neutralized, after exclusion of the liver, more CO₂ would be liberated from its combination in the tissues and the blood, and would escape. This is the explanation adopted by Rolly, and approved of by Fischler and Grafe. Rolly has actually found in the serum of his operated animals a lower degree of alkalescence than in that of normal animals and Porges in a recent paper⁹ has himself shown that acidification of the blood by intravenous infusion of sodium dihydrogen phosphate will raise the respiratory quotient, though not so much as occurred in his earlier experiments.

The mechanical factors have not been sufficiently emphasized.

⁶ Fischler and Grafe: *Deutsch. Arch. f. klin. Med.*, cviii, p. 516, 1912.

⁷ Böhm: *Zentralbl. f. Physiol.*, xxvii, p. 120, 1913.

⁸ Except one animal which had convulsions in Fischler and Grafe's series. Böhm's complete paper is not accessible and it is possible that his series may contain other exceptions.

⁹ Porges: *Biochem. Zeitschr.*, xlvi, p. 1, 1912.

Porges, in his original article assumes that any change due to over-ventilation which might result would be equalized in fifteen minutes. Presumably he means by over-ventilation only exaggerated breathing for he cites in support of his view, the work of Bornstein and Gartzten¹⁰ on the effects of over-ventilation by voluntary effort in human subjects, showing that *after fifty minutes no more CO₂ can be pumped out in this manner!* He also cites one of his own experiments in which the R. Q. in the second period was slightly higher than in the first period after ligation of the vessels. According to Porges' view, the quotient in the second period should be smaller if any factor of over-ventilation were operative.

Neither of these citations offers any convincing evidence which would exclude the mechanical factors; for in the experiments of Bornstein and Gartzten the circulation was in no way disturbed, while the forced breathing was maximal, and Porges' own experiment proves only that, whatever was the controlling cause of the higher quotient after ligation of the vessels, the conditions were the same in the second period as in the first. Moreover it should be borne in mind that over-ventilation may mean something more than exaggerated breathing: there may be over-aëration due solely to a disturbance to the pulmonary circulation.

Fischler and Grafe appreciated the possible effect of the disturbance to the circulation resulting from the Porges procedure, saying, "One does not know to what extent the results may be due to the direct consequences of this alteration."¹¹

In the writers' opinion the work of Fischler and Grafe is sufficient refutation of the position taken by the von Noorden school as to the rôle of the liver in the metabolism of the food-stuffs. Excluding the liver by ligation of the hepatic artery after Eck fistula *did not cause a permanent rise in the respiratory quotient* in dogs which survived from six to twenty hours. On the other hand, there is no doubt, in certain cases at least, about the rise of quotient after ligation of the abdominal aorta and inferior vena cava just below the diaphragm. It remains to give a satisfactory explanation of this phenomenon.

It seems almost incredible that the purely mechanical effects of so radical a procedure as one which excludes at a stroke fully

¹⁰ Bornstein and Gartzten: *Pflüger's Archiv*, cix, p. 628, 1905.

¹¹ Fischler and Grafe: *loc. cit.*, p. 519.

one-half of the blood and considerably more than one-half of the animal's weight from the circulation should not have been more seriously considered. What effect would it have on the heart rate, on the blood pressure, on the rate of blood flow through the lungs? Neither Porges nor Porges and Salomon gives any data as to the pulse, blood pressure, rate of respiration or volume of respiration, to say nothing of the gaseous content of the blood before and after ligation of the vessels, and yet the results are presented as proof that, by turning a valve, so to speak, the metabolic processes are suddenly changed so that one fuel and one only can now supply the body's energies!

The first and most obvious control which one would think of in connection with so radical a change in the R. Q. would be the gaseous content of the blood. There are numerous experiments in the work of Zuntz,¹² Krogh,¹³ Barcroft,¹⁴ Henderson¹⁵ and others showing that the O₂ and CO₂ contents of the blood are subject to considerable variations, particularly under operative conditions.

It is a commonplace laboratory exercise to clamp off the abdominal aorta below the diaphragm and witness the enormous rise in systemic pressure (carotid) which results. Simultaneous clamping of the inferior vena cava with the aorta will likewise produce the rise in systemic pressure. But if the two vessels are not clamped simultaneously, the mechanical effect will depend on the order in which the two are clamped. Clamping the inferior vena cava without clamping the aorta will produce a great fall in blood pressure for very obvious reasons. Hence, if the vena cava be clamped even as much as fifteen seconds before the aorta, the rise in carotid pressure is not so great as when the two are clamped together. *Vice versa* should the aorta be clamped first and even a small interval of time intervene before the vena cava is clamped, the blood from the abdominal viscera will continue to flow into the thorax until the *vis a tergo* is exhausted and the pressure on clamping the aorta will mount even higher, oftentimes so high as to cause heart failure.

These mechanical effects must, of necessity, affect the circula-

¹² Zuntz, N.: *Deutsch. med. Wochenschr.*, 1892, p. 109.

¹³ Krogh: *Skand. Arch. f. Physiol.*, xxiii, p. 179 *et seq.*, 1910.

¹⁴ Barcroft: *Ergob. d. Physiol.*, vii, p. 699, 1908.

¹⁵ Henderson: *Amer. Journ. of Physiol.*, xxi, p. 126, 1908; xxiv, p. 66, 1909.

tion through the lungs, the aëration of the blood in other words, and in turn the exchange of oxygen and carbon dioxide between blood and outside air.

Suppose the two vessels be clamped exactly at the same moment. If the heart remains competent to empty itself against the increased pressure, the blood will of necessity circulate more rapidly through the lungs, unless the heart compensates by beating more slowly. On the other hand, if the heart be not competent, it may go into fibrillations or beat imperfectly, in which case blood will accumulate in the lungs, producing passive congestion.

The former set of conditions should result in a decrease in the carbon dioxide in the blood because, the blood being exposed more often to the alveolar air, the carbon dioxide has more opportunity to escape. The latter set of conditions should result in an interference with oxygen absorption with or without a decrease in the carbon dioxide.

EXPERIMENTAL PART.

Reasoning along these lines, the writers have undertaken to determine to what extent clamping of the abdominal aorta and inferior vena cava would alter the carbon dioxide content of the blood as it leaves the heart (carotid artery).

Method.

The method of procedure in the earlier, orienting experiments was as follows. Normal dogs were anaesthetized with chlorotone. Urethane, which Porges and Porges and Salomon employed, has been avoided because it has been the experience in this laboratory that this drug excites the respiratory center of dogs much more than does chlorotone. Morphine has likewise been avoided, except in one experiment, because it tends to increase the CO_2 in the blood.¹⁶

When anaesthesia was fully established, the abdominal incision was made, the abdominal aorta exposed just above the origin of the coeliac axis, and hemostatic clamps were adjusted all ready to be closed at a signal. Before clamping, the pulse and respiration were usually counted and the control sample of blood was

¹⁶ Cushny: *Textbook of Pharmacology*, 1910, p. 221.

drawn. By these precautions one had knowledge of the condition of the animal just before the crucial operation. Then at a signal the two clamps were closed simultaneously, the closure of the vessels being immediately verified by examination. After the lapse of a varying interval of time, during which the pulse and respiration were counted frequently, the second sample of blood for analysis was drawn in the same manner as the first.¹⁷

The blood analyses were made by the chemical method of Haldane¹⁸ using the apparatus devised by Brodie.¹⁹ All determinations were made in duplicate.

The results of the preliminary experiments on five animals are presented in Table I.²⁰ It is evident, from an examination of these results, that a very great change in the aëration of the blood is brought about by the occlusion of these vessels. How serious a matter this change is for the life of the animal is seen in the fact that Dog III survived for only twenty minutes after the obstruction was accomplished. In all probability death was due to failure of the left ventricle.

Out of the four experiments in which a second analysis of blood was made, three exhibit a marked fall in the carbon dioxide of the arterial blood. Two show, in addition, a material fall in the oxygen content.

It is not to be supposed that the blood alone loses carbon dioxide. Arterial blood, containing less than the usual percentage of CO_2 , will carry away CO_2 by diffusion and this will continue the more rapidly the more the tension in the tissues exceeds that of the

¹⁷ The clamps which have been used for obstructing the vena cava are those known to surgeons as gastero-enterostomy clamps, fitted with rubber. Much difficulty has been experienced in placing a clamp on the aorta *above* the origin of the coeliac axis without serious rupture of the diaphragm and a number of animals were killed prematurely in this way. After this experience, however, it was found that by exposing the coeliac axis itself and just above it applying the clamp in such a way as to include the arcuate fibres about the aorta within the clamp it was possible to effect a complete obstruction without injury to other structures. Later a heavy wrapping cord was passed about the aorta at this point by means of a ligature carrier.

¹⁸ Haldane: *Journ. of Physiol.*, xxii, p. 465, 1897-8; Haldane and Barcroft: *Ibid*, xxxii, p. 232, 1902.

¹⁹ Brodie: *Ibid*, xxxix, p. 391, 1910.

²⁰ Cf. *Proc. Soc. Exp. Biol. and Med.*, x, p. 174, 1913.

arterial blood. The total amount of extra carbon dioxide appearing in the respiration after the clamps are applied, therefore, will depend upon the amount of the gas stored in the tissues. There is no perfectly satisfactory method of estimating the total carbon dioxide stored in the body at one time; hence it is impossible, from the percentages in the blood, to say just how much would escape in a given time. There is scarcely any doubt, however, that sufficient CO_2 has escaped from the animal's body in each of the three experiments, to cause a considerable rise in the respiratory quotient had it been determined for the period during which the vessels were clamped.

Respiration experiments.

Proof of the correctness of this view could only be had by repetition of the experiments of Porges accompanied by blood-gas analyses. Should the carbon dioxide of the arterial blood fall as in the foregoing experiments, during a respiration period occurring immediately after clamping of the vessels, and showing a higher R. Q., the conclusion would be irresistible that the higher quotient was due simply to an alteration in the rate of discharge and not an alteration in the rate of production of this gas. Again if the factor of over-ventilation, in the sense of increased breathing, were a controlling one, a period of exaggerated breathing preceding the clamping off of the vessels should nullify the effect of clamping on the respiratory quotient. It was desired also to make blood-pressure determinations before and after clamping to ascertain if possible what change is produced in the rate of flow through the lungs.

Method of respiration experiments.

The apparatus used was a respiration incubator constructed for the special purpose of studying the respiratory metabolism in new-born infants. It consists of a copper chamber ($30 \times 32 \times 76$ cm.) placed inside a Freas electric incubator,²¹ by means of which the chamber can be kept at a constant temperature, and connected

²¹ The Freas incubator can be purchased from Eimer and Amend, New York. The assemblage of apparatus as used in these experiments will be described in detail soon.

to a small Benedict²² respiration machine, by means of which it is ventilated.

The chamber will accommodate a dog of 6-10 kgm. The entire cubic contents of the air circuit is about 80 liters and, with the subject inside, the air space is correspondingly less. This fact permits of the determination of the R. Q. by the well-known method of Benedict²³ with an unusual degree of accuracy; for the reason that a small variation of temperature or of barometric pressure makes but a slight error in the oxygen determination. By making residual analyses at the beginning and end of each respiration period, even these errors may be eliminated. The apparatus has been thoroughly tested by burning alcohol inside it, with results, for the R. Q., very close to the theoretical value (0.666); namely, 0.661, 0.654, 0.662, 0.667.

For the blood-pressure readings the pulse-pressure instrument of Dr. C. J. Wiggers,²⁴ who very kindly instructed us in its use, was employed. Instead of the usual levers for graphic records, the maximal and minimal pressure tubes were connected directly to long mercury manometers and the maximum and minimum pressures were read off the millimeter scale directly. It was necessary to have unusually long manometers on account of the very great rise in pressure which often, though not always, takes place on obstructing the vessels. In one of the earlier experiments of this series the mercury was blown out by the excessive pressure and some 50 cc. of blood escaped from the carotid artery in the confusion which followed. The pressure readings were satisfactorily obtained in only two experiments. In order to obtain the true pulse pressure and not the maximo-minimal pressures²⁵ the readings were made while the respirations of the animal were inhibited temporarily by central stimulation of the vagus nerve.

All of the animals were anaesthetized with chloretone (usually given by stomach) and were tied lightly on an ordinary laboratory dog-board which had been sawed off to fit the respiration chamber. Once the dog is in the chamber and the latter sealed air-tight

²² Benedict, F.G.: *Amer. Journ. of Physiol.*, xxiv, p. 345, 1909.

²³ U.S. Dept. of Agriculture, Office of Experiment Stations, Bulletin 175, 1907.

²⁴ Wiggers, C.J.: *Amer. Journ. of Physiol.*, xxx, p. 233, 1912.

²⁵ Wiggers, C.J.: *loc. cit.*

the respiration experiment begins (after a short preliminary period of 10 to 25 minutes during which the chamber is being ventilated by the Benedict machine) on the second of the minute, by throwing a switch and turning a valve excluding the absorbers. Oxygen is admitted automatically throughout the period, but at the end the pressure is brought to the starting-point pressure by hand. The period was usually one hour in length.

I. Experiment on depancreatized dog showing higher R. Q. The first subject of this series was a depancreatized dog (Table II). That the animal was thoroughly diabetic is seen by the R. Q. obtained in two successive hours at the beginning of the experiments. The blood sample drawn at 3.12 p.m., just three minutes before clamping the vessels, showed a rather low percentage of both gases. It is well known that in diabetic subjects the CO_2 tension falls at times to a very low point.²⁶ Another sample drawn fifteen minutes after clamping however shows both gases still farther reduced—the carbon dioxide more than 10 per cent. Twenty-five minutes after taking this sample of blood the second respiration experiment began and continued for nearly two hours. The R. Q.s are decidedly increased, although the total respiratory exchange is very much reduced. *Accompanying the higher R. Q. is a very great depression in the carbon-dioxide content of the blood.* In view of the elevated blood pressure, which continued for twelve minutes at least after clamping the vessels, and the increased percentage of oxygen it seems likely that the true explanation of the lower percentage of CO_2 and hence of the higher R. Q. in this case is an increased aëration of the blood by more rapid circulation through the lungs.

II. Experiment in which the R. Q. remained the same after clamping the vessels. This was a normal dog, anaesthetized as usual. Blood pressures were determined before the respiration experiment. The dog had been fed the day before on dog biscuit, containing a high percentage of carbohydrate, and may have eaten some of it left over from the previous day, on the morning of the experiment. The R. Q. is rather high (probably for this reason) the first hour, but fell the second hour to a point more nearly within the range of a true *nüchtern* value.

²⁶ Beddard, Pembrey and Spriggs: *Lancet*, 1903, I, p. 1366.

Upon clamping, the blood gases fell rapidly within the next fifteen minutes and the blood pressures which were high at first fell rather suddenly to very low levels. *The R. Q. did not rise in the second respiration period and the CO₂ did not fall as in the previous experiment.* The explanation of the low blood pressure was found at autopsy in the fact that the clamp on the aorta had caught a bit of the stomach and for this reason was not quite competent to hold the arterial pressure. It was possible, after sectioning the aorta below the clamp, to squeeze blood through. The animal therefore must have bled into his abdominal vessels until the arterial pressure reached a level which could no longer pass the obstruction.

This experiment proves the relatively greater importance of CO₂ than of O₂ in the blood in determining the external R. Q. Oxygen is not stored in the tissues in any quantity as is carbon dioxide; consequently a considerable change in the O₂ content of the circulating medium does not affect the R. Q. materially.

III. Experiments in which the R. Q. fell after clamping the vessels. In the next experiment (Table IV) the dog exhibited a high R. Q. in the first period but instead of falling as is usual the further the time from feeding, it rose. Unfortunately the respiration rate was not recorded during these preliminary periods. It must be supposed however that there had been a considerable over-ventilation of the lungs and a consequent *Auspumpung* of CO₂; for upon clamping the vessels there was no fall, to speak of, in the CO₂ content of the blood within the first twenty minutes, and during the subsequent respiration period the CO₂ rose to 54.8 per cent while the oxygen fell. Again the CO₂ proves to be the determining factor; for its rise in the blood denotes a very considerable storage in the tissues and it is the holding back of this CO₂ which causes the R. Q. to fall to the extremely low level of 0.61 in the second period. The respiration apparatus was thoroughly tested immediately after this experiment and proved to be absolutely correct, giving a R. Q. with the alcohol flame of 0.667.

The results of the preceding experiments are fully confirmed in the following one (Table V) which was more complete. The dog had had no food since the previous day. The respiration rate was recorded during the preliminary respiration periods and established the cause of the high R. Q.s unquestionably to be the

Auspumpung of CO_2 . The temperature of the respiration chamber during these periods was very close to the critical temperature at which dogs begin to pant. This fact together with a rather light state of anaesthesia probably accounts for the high rate of breathing. *The pumping out of CO_2 in this case was so complete that upon clamping the vessels there was no reduction of the CO_2 in the blood, but instead a slight rise.* In all probability this rise started from a still lower level the moment the dog was removed from the respiration chamber, for the respiratory rate fell at once to normal. *In the respiration experiment which followed clamping of the vessels the R. Q., instead of rising, fell to an abnormally low point.* The clamps were absolutely competent.

Severe congestion of the lungs with oedema was found at autopsy, a circumstance which explains the very low percentage of oxygen found at the end. The carbon dioxide was not so high, however, as in the previous experiment. The rate of respiration declined rapidly and the dog was near death when removed from the chamber.

One other experiment, not reported in detail, was performed on a normal fasting dog, in which the R. Q.s in the preliminary periods were 0.72 and 0.85, while after clamping it was 0.67. The same explanations probably apply.

Two other experiments on depancreatized dogs were attempted but both died upon clamping of the vessels. Porges and Salomon succeeded in obtaining respiratory periods after ligation of the vessels in only four depancreatized dogs out of fifteen. There are obvious reasons why the animals do not survive longer. The strain upon the heart is tremendous. In several dogs, both normal and depancreatized, of this series, the heart failed at once and could not be revived. Aside from this the very rapid fall in the CO_2 percentage, which cannot be entirely compensated for by reduced rate (see Dog V, Table I) must produce a profound effect on all the higher brain centers. When, added to this, we consider that the congestion of the lungs is such as to interfere with the absorption of oxygen, the wonder becomes that so many animals survive as long as they do.

Alkalinity of the blood.

Rolly²⁷ has established, by a new and much improved method, the fact that in dogs operated after the Porges procedure, the H-ion concentration of the blood is increased and the OH-ion concentration is diminished. This observation has been confirmed in a single examination of the blood reaction made in these experiments. From Dog IX, 20 cc. of carotid blood were drawn (10 cc. into each of two centrifuge tubes containing 0.5 cc. each of 0.1 per cent hirudin solution) before clamping the vessels and again just after drawing the last sample of blood for gas analysis. Ten cc. of the hirudin plasma titrated to the first pink color of phenolphthalein with $\frac{N}{10}$ NaOH required for the first sample 4.2 cc. and for the second 7 cc. *The acidity*, in other words, *had nearly doubled*, and yet in spite of this change the CO₂ was held back coincidentally so as to reduce the R. Q. to 0.633! From this single observation the indications are that this greater acidity (H-ion concentration) cannot be the only cause of the extra elimination of CO₂.

DISCUSSION OF THE FACTOR OF EXAGGERATED BREATHING.

This series of experiments was undertaken in the full expectation of finding mechanical factors adequate to explain *any alteration* in the R. Q. which could result from sudden obstruction of the main vessels leading to and from the abdominal organs. One such factor, exaggerated breathing, unquestionably is; for in these experiments it has been shown (Dogs VIII and IX) that increased respiratory activity may keep the quotient far above normal for at least two hours. That over-ventilation (exaggerated breathing) was present in the experiments of Porges and of Porges and Salomon may be inferred, in the absence of direct data, from the expressed assumption of Porges that after fifteen minutes of exaggerated breathing no more CO₂ could be pumped out. Furthermore it is the experience of this laboratory that urethane, which Porges and Salomon used, always excites the respiratory center (in dogs) and that it cannot always be controlled with moderate doses of morphine. In a former series of experiments in which the respiration apparatus was attached directly to the

²⁷ Rolly: *Munch. med. Wochenschr.*, 1912, Nos. 22 and 23.

trachea²⁸ urethane was tried and was given up for this very reason. In the original experiments of Porges and of Porges and Salomon, the higher quotients are doubtless due in part to this form of over-ventilation.

DISCUSSION OF THE FACTOR OF BLOOD FLOW THROUGH THE LUNGS.

That some other factor than exaggerated breathing may account for a great reduction in the CO_2 of the arterial blood and therefore for a rise in the respiratory quotient after clamping of the vessels, is seen from the experiments with Dogs I, II and V (Table I) and Dog VI (Table II). In none of these experiments was any increased breathing observed. The blood-pressure determination with Dog VI gave a clue which it was hoped would lead to definite conclusions on the matter of blood-flow when the pulse pressures were more accurately determined in the experiments with Dogs VII and IX. Unluckily the leak in Experiment VII invalidated the blood-pressure findings, as a criterion of blood-flow in that experiment; for the mean pressure changed. In Experiment IX however it may be seen that the minute volume of blood-flow through the heart, and therefore through the lungs has changed greatly after clamping and that this change is consistent with the change in blood gases.

According to the law of von Recklinghausen²⁹ the amplitude of the pulse wave (pulse-pressure) at any given mean pressure is a measure of the systolic output, provided the distensibility of the arterial wall is constant. The product of the pulse-pressure by the pulse-frequency is then a measure of the minute-volume.

There is no reason to suppose that the distensibility coefficient *per se* of the arterial system is in any way altered by the clamping of the aorta and vena cava. Therefore if the mean pressure remains about the same the product of pulse-pressure into pulse-frequency would afford a criterion of the effect of the operation on the blood-flow.

Referring to Table V it is seen that the pulse pressure just before clamping is three and one-half times as great as just after clamping. The mean pressure has risen slightly but not sufficiently

²⁸ Murlin and Greer: *Amer. Journ. of Physiol.*, xxvii, p. xviii, 1911.

²⁹ v. Recklinghausen: *Arch. f. exp. Path. u. Pharm.*, lvi, p. 1, 1906.

to offset the difference in pulse pressure.³⁰ The pulse frequency is considerably higher before the operation than after it. Hence the blood-flow through the lungs has been greatly reduced by the operation. The surprising thing is that such a change in the blood-flow should not have produced a greater effect on the exchange of gases.

Two facts then stand out with some significance in the matter of blood-flow. In Experiment VI where the CO_2 in the blood fell rapidly after clamping of the vessels (while the O_2 rose), and the R. Q. as a consequence rose, the pulse pressure was maintained. Since there is no reason to believe that the pulse rate suffered any diminution (see Experiments I-V), the minute volume after clamping was at least as great as before. In Experiment IX where the CO_2 in the blood rose slightly (while the O_2 fell) and the R. Q. as a consequence was falling (after the previous over-ventilation) the minute volume was distinctly less. These two facts are offered not as final proof but as evidence, consistent as far as it goes, that

³⁰ v. Recklinghausen's formula is $A = \frac{R}{\left(\frac{dI}{dp}\right)_{\mu}} \times 1/k$ where A is ampli-

tude or pulse pressure, R is pulse volume, the expression $\left(\frac{dI}{dp}\right)_{\mu}$ denotes distensibility of the arterial wall, at the mean pressure and k is a constant determined by viscosity, diameter of vessels, etc. The pulse volume R then would be expressed by the formula $\frac{A (\text{distensibility})}{1/k}$.

Making substitutions from Table V the pulse volume before clamping would be $\frac{22 \times \text{distensibility at } 54}{1/k}$; after clamping it would be

$$\frac{6 \times \text{distensibility at } 72}{1/k}.$$

Supposing the distensibility and the value of k to be the same the pulse volume before clamping is more than three times the value after clamping. The minute volume would be found by multiplying the value of the pulse-volume, or systolic output, by the pulse-frequency. Taking 210 as pulse-frequency just before clamping and 180 just after, the minute volume proves to be less than one-third its former value. In all probability this difference is too great; the point is to show that distensibility or the value of k would have to change a great deal to offset the difference in pulse pressure observed.

the altered rate of blood-flow through the lungs is an important factor in determining the CO_2 (and O_2) content of the blood and therefore in explaining the altered respiratory exchange.

CONCLUSION.

Whether one or both of the factors discussed above are controlling, there can be no doubt as to the significance of the blood-gas analyses. In each instance the blood-gas changes are consistent with the mechanical explanation of the altered respiratory quotients after clamping the vessels. Where the R. Q. rose (Experiment VI) the CO_2 of the blood fell; where the quotient remained stationary (Experiment VII), the CO_2 did not change; and where the R. Q. fell (Experiments VIII and IX), the CO_2 in the arterial blood rose. Clamping off the blood from the abdominal organs therefore does not alter the character of the metabolism, and the experiments of Porges and of Porges and Salomon have no bearing on the problem of the oxidation of sugar.

94 Metabolism after Clamping Abdominal Vessels

TABLE I.

Dog I. 8 kgm. March 22, 1913. Chloretone per rectum.

TIME	EVENT	PULSE	RESPIRATION	BLOOD ANALYSIS	
				O ₂	CO ₂
<i>p.m.</i>				<i>per cent</i>	<i>per cent</i>
3.20	4.3 cc. carotid blood drawn			15.43	43.6
3.25	Vessels clamped simultaneously				
3.30		96	36		
3.40		144	30		
3.45		138	30		
3.54	4.2 cc. carotid blood drawn			15.52	22.53
3.55	Clamps removed				
4.00		120	35		

Dog II. 12 kgm. April 10, 1913. Chloretone intraperitoneally.

2.15	4.4 cc. carotid blood drawn			17.10	38.35
2.17	Vessels clamped simultaneously	66	35		
2.20		120	24		
2.35		102	12		
2.40		102	30		
2.41	3.2 cc. carotid blood drawn			17.16	37.47
2.47	Clamps removed				

Dog III. 7.5 kgm. April 12, 1913. Chloretone anaesthesia.

2.08		108	30		
2.20		114	24		
2.30		114	24		
2.35		120	15		
2.36	4.5 cc. carotid blood drawn			18.85	42.01
2.40		120	24		
2.46	Vessels clamped simultaneously; heart stopped				
2.58			24		
2.59	Artificial respiration				
3.00		96	30		
3.08		120	54		
3.10	Dog died; clamps on only 20 minutes; cause of death not apparent				

TABLE I.—Continued.

Dog IV. 9 kgm. April 19, 1913. Chloretone by stomach.

TIME	EVENT	PULSE	RESPI- RATION	BLOOD ANALYSIS	
				O ₂	CO ₂
<i>p.m.</i>				<i>per cent</i>	<i>per cent</i>
1.50		138	35		
2.25		120	72		
2.32	4.4 cc. carotid blood drawn	138	66	19.52	39.42
2.38		132	60		
2.40	Vessels clamped simultaneously				
2.43		104	96		
2.52		126	64		
3.02		120	78		
3.15		120	80		
3.25		120	72		
3.35		126	72		
3.43	4.35 cc. carotid blood drawn			17.09	24.28
3.44	Clamps removed				
3.45		120	60		

*Dog V. 10 kgm. May 10, 1913. Morphine subcutaneously.
Chloretone by stomach.*

2.28		96	34		
3.15		120	32		
3.17	4.15 cc. carotid blood drawn			13.32	51.06
3.20	Vessels clamped simultaneously				
3.22		102	16		
3.27		120	16		
3.33		120	14		
3.43		120	14		
4.03		120	14		
4.13		120	18		
4.18		120	16		
4.22	4.3 cc. carotid blood drawn			11.60	34.16
4.23	Clamps removed	120	24		
4.29		108	24		
4.39		108	24		

TABLE II.

Dog VI. 6.1 kpm. Depancrutilized July 7, 1913. Experiment July 9, 1913. Chlorelone by stomach.

TIME	EVENT	BLOOD PRESSURE		PULSE PER MIN.	RESP. PER MIN.	BLOOD ANALYSIS		RESP. CO ₂	METAB. O ₂	R. Q.	TEMP. OF RESP. APP.
		Min.	Max.			O ₂	CO ₂				
a.m.											deg. C.
11.27-12.27	In respiration apparatus							grams	grams	0.68	33.50
p.m.											
12.27-1.27	In respiration apparatus										
1.30-2.30	Operation and arrangement of blood pressure apparatus									0.68	34.0
3.00		70	80								
3.12	5.6 cc. blood drawn	66	84			11.06	34.72				
3.15	Vessels clamped simultaneously										
3.16		148	172								
3.17		130	174								
3.20		80	92	106	24						
3.27		80	94	112	28						
3.30	4.33 cc. blood drawn					9.85	24.07				
3.35	Returned to respiration apparatus										
3.55-4.55	Respiration experiment							2.35	2.07	0.83	30.0
4.55-5.42	Respiration experiment							1.74	1.55	0.82	30.4
5.45	4.6 cc. blood drawn										
6.00	Autopsy; clamps competent; no congestion of lungs					13.70	12.98				

TABLE III.
Dog VII. 9.5 kgm. Normal, July 29, 1913. Chloretone by stomach.

TIME	EVENT	PULSE	RESP.	BLOOD PRESSURE		BLOOD ANALYSIS		RESP. CO ₂	METAB. O ₂	R. Q.	TEMP. OF RESP. APP.
				Min.	Max.	O ₂	CO ₂				
<i>a.m.</i>						<i>per cent per cent</i>		<i>grams</i>	<i>grams</i>		<i>deg. C.</i>
10.20	Exposed carotid	150	22								
10.24	Tied left vagus										
10.24	Cut left vagus	140	16								
10.25											
10.26				116	134						
				112	132						
				116	126						
10.27	Left vagus stimulated centrally	126		108	124						
10.28	Left vagus stimulated centrally	126		94	110						
10.29	Left vagus stimulated centrally	132		86	110						
10.30	Placed in respiration apparatus										
10.55-11.40	Respiration experiment		18					4.630	3.897	0.864	32.6
11.40-12.25	Respiration experiment		28-40					4.537	4.069	0.812	32.9
<i>p.m.</i>											
12.27	Laparotomy										
12.31		142	50								
12.46	Left vagus stimulated centrally	150		80	94						
12.47	Left vagus stimulated centrally	132		80	96						
12.48	Left vagus stimulated centrally	108		76	94						
12.50	4.4 cc. blood from carotid					25.73	37.38				
1.00				64	74						
1.03	Vessels clamped simultaneously			120	146						

TABLE IV.
Dog VIII. 5.5 kgm. Normal. July 26, 1913. Chloroform intraperitoneally.

TIME	EVENT	PULSE PER MIN.	RESP. PER MIN.	CAROTID BLOOD ANALYSIS		RESP. CO ₂	EXP. O ₂	R. Q.	TEMP. OF RESP. APP.
				O ₂	CO ₂				
				per cent per cent		grams	grams		deg. C.
a.m.									
10.35-11.35	Respiration experiment					4.512	4.096	0.861	31.0
11.35-12.35	Respiration experiment					4.859	3.756	0.941	31.1
p.m.									
12.40	Dog operated	132	48						
12.49		154	56						
1.05									
1.08									
1.10	4.6 cc. blood drawn								
1.12	Vessels clamped simultaneously	140	68	23.22	44.31				
1.27		140	52						
1.30	4.4 cc. blood drawn			20.48	43.95				
1.33	Returned to apparatus								
1.55-2.55	Respiration experiment		64			1.280	1.314	0.709	30.0
2.55-3.50	Respiration experiment		58-24			0.88	1.05	0.61	29.6
3.55	3.8 cc. blood drawn			7.1	54.80				
4.00	Dog died; autopsy showed clamps competent; slight congestion of lungs								

TABLE V.
Dog IX. 6.5 kgm. Normal. August 1, 1913. Chloretone by stomach.

[illegible]

2.24	Left vagus cut and stimulated	210							
2.28	Left vagus stimulated	220		50	70				
2.30	3.4 cc. carotid blood drawn			42	62				
2.31	20 cc. carotid blood drawn for alkalimetry test; 10 cc. titrated 4.2 cc. $\frac{N}{10}$ NaOH					10.72	35.36		
2.35	Vessels clamped simultaneously								
2.41	Left vagus stimulated centrally	180		70	76				
2.42	Left vagus stimulated centrally	180		70	76				
2.43			42						
2.46	Left vagus stimulated centrally	192		68	74				
2.50	4.5 cc. carotid blood drawn					lost	37.27		
2.52	Dog in respiration apparatus								
3.08	Third period begins								
3.15			28						
3.30			28						
3.40			16						
3.55			18						
3.58	Third respiration period ends								
4.02	Dog out of respiration apparatus								
4.03	4.5 cc. carotid blood drawn								
4.04	20 cc. carotid blood drawn for alkalimetry; 10 cc. plasma titrated 7.0 cc. $\frac{N}{10}$ NaOH								
4.10	Dog died. Autopsy showed oedema of both lower lobes, clamps entirely competent					4.6	39.36		
								1.896	2.181 0.633 31.9

THE SEPARATION OF *d*-ALANINE AND *d*-VALINE.

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In the ester method of protein hydrolysis the esterifiable amino-acids are separated by distillation into two fractions, a higher boiling containing aspartic and glutaminic acids, phenyl alanine, and serine, and a lower boiling fraction containing proline, *l*-leucine, *d*-isoleucine, *d*-valine, *d*-alanine, and glycocoll. For several years we have been trying to devise methods to approximate as nearly as possible a quantitative separation or determination of the six amino-acids composing the latter mixture.

Proline, unlike the other members of this fraction, is very soluble in alcohol,¹ and is partially separated from them by alcoholic extraction. The extract, however, usually consists of about two-thirds proline and one-third of a mixture of other amino-acids which have gone with the proline into solution in the alcohol. Proline, however, contains no primary amino nitrogen, while all the nitrogen of the other acids of this ester fraction is in the form of primary amino groups. Therefore, a determination of the total and the primary amino nitrogen,² respectively, in the extract permit one to calculate accurately the amount of proline, which is indicated by that of the non-amino nitrogen.

The other five amino-acids can be distributed by fractional crystallization among subfractions the composition of which varies greatly according to the proportions in which the different acids are present. As glycocoll and alanine dissolve at room temperature in only four parts of water, while the other three, particularly

¹ Fischer: *Ber. d. deutsch. chem. Gesellsch.*, xxxix, p. 530, 1906.

² Van Slyke: Quantitative Determination of Proline obtained by the Ester Method in Protein Hydrolysis, this *Journal*, ix, p. 205, 1911; Quantitative Determination of Aliphatic Amino Groups, this *Journal*, ix, p. 185, 1911 and xii, p. 275, 1912.

the leucine and isoleucine, are much less soluble, one can usually obtain by crystallization the greater part of the mixture in two fractions, a comparatively insoluble one consisting of the leucine and isoleucine, together with much of the valine, and a very soluble fraction containing glycocoll and alanine. For the quantitative determination of the proportions in which leucine, isoleucine, and valine are present in the less soluble fraction we have already published methods which have been utilized with satisfactory results.³ More recently we have described the separation of glycocoll from alanine in the more soluble subfraction by means of glycocoll picrate, which is difficultly soluble in cold water.⁴

Besides the leucine-isoleucine-valine and the glycocoll-alanine crystallized fractions, however, one usually obtains another, intermediate between these two, containing alanine and valine in such proportions that they cannot be separated by crystallization. This paper presents a method for the separation of the alanine and valine of this intermediate fraction. One can now determine all the six amino-acids from the lower boiling ester fraction with a fair degree of accuracy. This does not mean that they are completely regained in the amounts in which they are present in the proteins. Losses which prevent this still occur in the esterification and distillation of the esters. The uncertainties, however, which were formerly connected with the separation of these amino-acids after the distillation, are now reduced to comparatively small proportions.

We have determined the following data, on which is based the method for separating valine from alanine, and from glycocoll in case this also should occur in the intermediate fraction.

Data on which the separation is based.

d-Alanine in the presence of 10 per cent sulphuric acid is precipitated by phosphotungstic acid as a crystalline salt which contains approximately 14 parts of phosphotungstic acid to 1 of

³ Levene and Van Slyke: this *Journal*, vi, p. 391, 1909. Abderhalden and Weil have recently isolated from nerve tissue a third leucine isomer. We did not find evidence of it in casein or edestin; but if it proves to be a general constituent of the proteins still further development of special methods for this fraction will be necessary. *Zeitschr. f. physiol. Chem.*, lxxxiv, p. 39, 1913.

⁴ Levene and Van Slyke: this *Journal*, xii, p. 285, 1912.

alanine. At 0° about twenty-four hours are required for precipitation of the maximum amount of alanine. The presence in solution of about 20 grams of phosphotungstic acid (in excess of the amount precipitated with the alanine) per 100 cc. of solution is required to insure most complete precipitation. Under these conditions the amount of alanine left in solution at 0° in 100 cc. of mother liquor is 0.15 gram. The concentration of free phosphotungstic acid can be increased up to at least 70 grams per 100 cc. of solution without either increasing or diminishing to a significant extent the solubility of alanine phosphotungstate.

d-Valine has under the same conditions the much greater solubility of 1.2 grams per 100 cc. Valine phosphotungstate shows, under proper conditions, very little tendency to form mixed crystals with alanine phosphotungstate. In case a mixture of the two is obtained, one can readily separate them by recrystallization from a solution containing 10 per cent of sulphuric and 20 per cent of phosphotungstic acid.

The solubilities of the phosphotungstates of both alanine and valine are very dependent upon the concentration of sulphuric acid present.

Glycocoll is precipitated under the same conditions as *d*-alanine, only 0.2 gram of glycocoll remaining in 100 cc. of mother liquor.

Lead acetate, recently recommended by Benedict and Murlin⁵ for the removal of phosphotungstic acid from solutions containing amino-acids, is the most satisfactory reagent which we have found for freeing both alanine and valine from sulphuric and phosphotungstic acids. The precipitation of phosphotungstic acid is quantitative, and the small amount of lead sulphate remaining dissolved in the filtrate is readily removed by addition of an equal volume of alcohol. Five per cent, and sometimes even more, of the amino-acid present are usually adsorbed by the heavy precipitate, but the loss is less than when barium hydrate is used, and the amino-acid regained after removing the excess lead as sulphide and concentrating the solution to dryness contains less than 1 per cent of ash.

Natural leucine is precipitated by concentrated solutions of phosphotungstic acid, the precipitate being redissolved by sufficient excess of the acid, as found by Levene and Beatty. Leucine

⁵ *Proc. Soc. Exp. Biol. and Med.*, 1912.

may interfere with the purification of alanine as the phosphotungstate, however, and should be removed, either by crystallization or by precipitation as the lead salt⁶ before the separation described below is begun.

Dilute methyl and ethyl alcohol are unsuitable solvents for the recrystallization of valine when even a small proportion of alanine is present; because the relative solubilities of the two amino-acids in water are reversed in both alcohols, in which alanine is much less soluble than valine. This is the case to a less marked extent with acetone, and it is, therefore, better suited to throw valine out of water solution in the presence of alanine. If to 100 cc. of water at 20° one adds 200 cc. of 80 per cent acetone, the resulting solution will dissolve 3.2 grams of alanine and 3.4 of valine. The solubility relations are such that one can add 3, 4, 5, 6, or 7 volumes of 80 per cent acetone with nearly the same effect. A mixture of 100 cc. of water and 700 cc. of 80 per cent acetone dissolves 2.5 grams of alanine and 3.4 of valine. Consequently, as the results are within a wide range independent of the volume of solution added, 80 per cent acetone affords a convenient means for throwing valine out of water solution in the presence of small amounts of alanine.

Because of the fact that alanine is much less soluble than valine in ethyl and methyl alcohol, especially the latter, it was thought that valine could, perhaps, be extracted from a mixture of the two amino-acids by means of methyl alcohol. It was found, however, that it was impossible to extract all the valine without also dissolving a large proportion of the alanine.

Precipitation and purification of alanine as phosphotungstate.

The mixture of valine and alanine should preferably contain not over 50 per cent of valine. If more is present, part can readily be removed by recrystallizing from water, in which valine is much less soluble than alanine.

It is advisable, because of the appreciable solubility of alanine phosphotungstate, to precipitate it from as small a volume of 10 per cent sulphuric acid as will hold the valine in solution. In order to obtain at once alanine phosphotungstate free from valine

⁶ Levene and Van Slyke: this *Journal*, vi, p. 391, 1909.

the volume of solution must be as great as 100 cc. for each gram of valine present. If the alanine phosphotungstate is recrystallized, however, one need use but 30 to 40 cc. for each gram of valine, recrystallizing once from a similar volume of fresh solution. One thus completes the separation, using in all only 60 to 80 per cent of the volume of solution required when one does not recrystallize, and one is also somewhat more certain of the absolute purity of the alanine. The process which gives the most satisfactory separation is the following:

The mixture of alanine and valine is dissolved in a hot solution which contains 10 grams of sulphuric acid per 100 cc. The volume of this 10 per cent sulphuric acid used should be 30–40 cc. for each gram of valine which analysis of the mixture indicates can, as a maximum, be present. In the hot solution one further dissolves enough purified phosphotungstic acid to combine in the ratio of 14:1 with the maximum amount of alanine which previous analysis has indicated can be present in the mixture, and in addition leave 1 gram of excess phosphotungstic acid for every 5 cc. of the 10 per cent sulphuric acid used. The use of a greater excess of phosphotungstic acid does not interfere with the separation, but leaves one an unnecessarily large amount to remove at the end of the operation. The solution prepared as above directed is placed in a refrigerator at 0° and allowed to remain there for at least twenty-four hours.⁷ In case the volume of the solution is large, time must be allowed for it to cool before beginning to count the period allowed for crystallization. The precipitate separates in large, transparent crystals, which form a solid layer about the walls and bottom of the flask. When sufficient time has been allowed for the separation, the supernatant solution is decanted off as completely as possible. The crystals are then redissolved by heating with a volume of 10 per cent sulphuric acid equal to that originally used. Phosphotungstic acid, in the ratio of 1 gram to each 4 or 5 cc. of 10 per cent sulphuric acid used, is then dissolved in the hot solution, and the alanine phosphotungstate is again allowed twenty-four hours at 0° to crystallize. The supernatant solution is again decanted, and the crystals are washed with suction with a small volume of an ice-cold solution containing 10 per cent of sulphuric and 20 per cent of phosphotungstic acid.

⁷ If only an ordinary ice box, which usually gives a temperature of 8°, is available, the flask should be immersed in ice water.

Determination and isolation of the precipitated alanine.

The alanine phosphotungstate is at once dissolved in hot water, where it forms a solution that is usually somewhat turbid. It is diluted in a measuring flask to such a volume that 10 cc. contain from 50 to 100 mgms. of alanine, and aliquot parts are used for determination of the nitrogen present. The determination is most conveniently performed by the nitrous acid method for determination of amino nitrogen.⁸ If the micro-apparatus (cf. p. 121) is used 2 cc. of solution are sufficient; with the larger apparatus one uses 10 cc. The determination can also be done according to Kjeldahl, although in this case it is necessary to draw air through the mixture, while it is digesting with sulphuric acid, in order to prevent the violent bumping which the precipitated tungstic acid causes.⁹ It is preferable to base the calculation of the amount of alanine present on the nitrogen determination rather than on the substance actually isolated, because, when the phosphotungstic acid is removed with lead, the bulky precipitate of lead phosphotungstate adsorbs several per cent of the alanine present, and the amount actually recovered is only 90–95 per cent of that in solution before the removal of the mineral acids. To the amount of alanine calculated from the nitrogen determination one may add a solubility correction for the amount dissolved in the total volume of solution from which the alanine was precipitated and recrystallized. This amount is calculated on the basis of a solubility of 0.15 gram of alanine per 100 cc.

The remainder of the solution, after the portion for the analysis has been removed, is washed into a Jena beaker and heated to boiling. A 20 per cent solution of neutral lead acetate is added in portions until an excess is present, and can be detected, by means of the sulphuric acid test, in a drop removed from the surface of the solution in the beaker. The heavy precipitate of lead sulphate and phosphotungstate is filtered with suction and washed thoroughly with water. The filtrate is concentrated to a volume

⁸ This *Journal*, ix, p. 185, 1911, and xii, p. 275, 1912. As there is so much mineral acid present, it is advisable to add, to the nitrous acid solution in the apparatus, enough 4 or 5 *N* NaOH to nearly neutralize the sulphuric acid before the solution containing the latter is run in.

⁹ Denis: this *Journal*, viii, p. 427.

of about 50 cc. for each gram of alanine present, and mixed with an equal volume of 95 per cent alcohol. This precipitates a small amount of lead sulphate which had remained, owing to its slight but appreciable solubility in water. The solution is allowed to stand on the water bath for an hour or more to complete the precipitation, the sulphate is filtered off, and the excess of lead in the filtrate is removed with hydrogen sulphide. The lead sulphide is washed with water through which H_2S has been bubbled, and the filtrate is concentrated, preferably in vacuum, to a small volume. It is then transferred to a Jena glass evaporating dish and the concentration continued on the water bath until all the visible liquid has been evaporated. The drying is completed in a vacuum desiccator over sulphuric acid and potassium hydrate. It is not advisable to try to drive off with heat the last traces of water and acetic acid, for this is likely to somewhat discolor the substance. The product, dried in vacuum, is perfectly colorless, nearly ash-free (if pure reagents have been used), and free from valine.

Besides the alanine isolated as above described, a small amount, left in solution when the alanine phosphotungstate was precipitated, is later obtained from the mother liquors of the valine.

In case the original valine-alanine mixture contained glycocoll, the latter will now be found with the alanine, from which it can be separated as the picrate, according to the method described by us.¹⁰

Determination and isolation of the valine.

The decanted filtrates and the washings from the alanine phosphotungstate are diluted to a definite volume and the amino nitrogen determined in an aliquot part, in the manner described for the alanine solution. A special blank determination to ascertain the correction for the reagents should be made, using as the control solution 10 per cent sulphuric acid instead of water, as the presence of so much mineral acid increases the correction. The phosphotungstic and sulphuric acids are removed with lead acetate, as in the isolation of alanine, and the valine solution, free from mineral acids and bases, is concentrated on the water bath until the valine begins to crystallize at the surface. Two or three

¹⁰ Levene and Van Slyke: this *Journal*, xii, p. 285, 1912.

volumes of 80 per cent acetone are then added, and the mixture is rinsed, using more 80 per cent acetone, into a flask. This is stoppered to prevent evaporation of the acetone, and allowed to stand over night while the valine crystallizes. The latter is filtered, washed with 80 per cent acetone, and thus obtained free from alanine in a yield of 80 to 85 per cent of the amount present.

The filtrate from the valine contains the small amount of alanine which escaped precipitation by phosphotungstic acid, and an amount, usually about equal, of valine, which remained in solution in the dilute acetone. The filtrate is concentrated to dryness, weighed, and the alanine and valine separated with phosphotungstic acid as before. This second crystallization makes the separation practically quantitative.

When refrigeration facilities do not enable one to keep the solutions at 0° during the entire period while the alanine is being precipitated, one can let the solutions stand over night at room temperature, and then place them in ice water for several hours, stirring them occasionally to complete the crystallization at 0°. The precipitation is nearly, though not quite, so complete as when the solution is kept at 0° for the entire period.

Working at room temperature entirely, one can precipitate at least 75 per cent of the alanine in purity, using one-half the volume of solutions given in the above directions.

Purity of reagents.

Because of the large amounts of lead acetate and phosphotungstic acid used, both reagents must be pure or the amino-acids obtained after their use will be accompanied by ash. The lead acetate should leave no residue after precipitation of a solution with hydrogen sulphide and evaporation of the filtrate to dryness. We have had no difficulty in obtaining good lead acetate from the manufacturers. The phosphotungstic acid should leave no residue after precipitation with pure lead acetate and evaporation of the filtrate. We purify the commercial phosphotungstic acid by Winterstein's method. The acid is dissolved in a small amount of water, from which it is shaken out with ether. With the latter it forms an oily solution much heavier than water. The ether solution is washed several times with water, and the ether is driven off on the water bath. The product is not hygroscopic, and forms a colorless solution.

EXPERIMENTAL.

Analysis of materials.

d-Alanine was obtained from hydrolyzed silk by the ester method. The glycocoll accompanying the alanine in the amino-acids obtained from the low boiling fraction of esters was removed with picric acid,¹¹ and the *d*-alanine was purified by recrystallization from dilute alcohol. It gave the following figures on analysis.

Substance, 0.1195 gram; CO₂, 0.1764 gram; H₂O, 0.0825 gram.

Substance, 0.0909 gram; nitrogen gas at 21°, 763 mm. (nitrous acid method), 25.20 cc.

Substance, 0.1817 gram; solution (containing 1.3 mols. HCl), 2.4910 grams; concentration, 7.29 per cent; sp. gr., 1.03; rotation in 2 dm. tube with yellow light from a spectroscope, $+2.07^\circ \pm 0.01^\circ$.

Substance, 0.1422 gram; solution in 20 per cent HCl, 2.5810 grams; concentration, 5.51 per cent; sp. gr., 1.087 at 25°; rotation in 2 dm. tube, $+1.64^\circ \pm 0.01^\circ$.

	Found:	Calculated for C ₃ H ₇ O ₂ N:
C.....	40.25	40.41
H.....	7.72	7.92
N.....	15.74	15.73
[α] _D with 1.3 mols. HCl....	+9.77°	+10.30° (Calculated for HCl salt.) ¹²
[α] _D with 1.3 mols. HCl....	+13.78°	Calculated for amino- acid.
[α] _D with 20 per cent HCl..	+9.72°	Calculated for HCl salt.
[α] _D with 20 per cent HCl..	+13.69°	Calculated for amino- acid.

From the above figures it is apparent that the *d*-alanine was analytically pure and as free from *dl*-alanine as one can usually prepare it from hydrolyzed protein. The rotation is, as stated by Emil Fischer, practically unaffected by the amount of excess hydrochloric acid present.

d-Valine was prepared from casein by esterification and the use of our lead method.¹³ The preparation gave the following figures on analysis:

Substance, 0.1203 gram; CO₂, 0.2260 gram; HO₂, 0.1013 gram.

Substance, 0.1081 gram; nitrogen, 22.9 cc. at 25°, 762 mm. (nitrous acid method).

¹¹ Levene and Van Slyke: this *Journal*, xii, p. 285, 1912.

¹² E. Fischer: *Ber. d. deutsch. chem. Gesellsch.*, xxxix, p. 464.

¹³ Levene and Van Slyke: this *Journal*, vi, p. 391, 1909.

Substance, 0.1510 gram; solution in 20 per cent HCl, 2.6240 grams; sp. gr., 1.10; rotation in 2 dm. tube with yellow light, $+3.28^\circ \pm 0.01^\circ$.

	Found:	Calculated for $C_6H_{11}O_2N$:
C.....	51.21	51.24
H.....	9.43	9.47
N.....	11.97	11.96
$[\alpha]_D^{20}$	$+25.93^\circ$	$+28.80^\circ$

The valine was analytically pure. The rotation was lower than that obtained by Fischer for synthetic *d*-valine,¹⁴ but is as high as one usually obtains in the natural product after acid hydrolysis. As the valine obtained by acid hydrolysis of proteins usually has a rotation of $+24^\circ$ to $+26^\circ$, the use of the above material gives one more nearly the conditions actually met in hydrolysis work than would employment of the optically pure synthetic substance.

Composition of alanine phosphotungstate.

Levene and Beatty found that alanine combines with phosphotungstic acid to form a crystalline salt.¹⁵ We have prepared the salt as nearly pure as possible in order to determine its composition. Preliminary preparations showed that the ratio of alanine to phosphotungstic acid was approximately 1:14. We dissolved the two constituents in this ratio (0.5 gram of alanine and 7 grams of phosphotungstic acid) in 15 cc. of normal hydrochloric acid, and let the solution stand over night while the salt crystallized. The crystals were filtered on a clay plate and dried over solid potassium hydrate until the chloride reaction disappeared. The product was further dried in a vacuum at 100° . The proportion of alanine was then determined by estimation of the amino nitrogen with nitrous acid. The results were:

N.....	1.036 per cent.
Alanine.....	6.57 per cent.
PTA.....	93.33 per cent.
Ratio, alanine: PTA.....	=1:14.1

The salt forms with water of crystallization. The air-dried substance loses 3.8 per cent of its weight when dried in vacuum at 100° , and the anhydrous salt when exposed to air takes up a sim-

¹⁴ *Ber. d. deutsch. chem. Gesellsch.*, xxxix, p. 2320.

¹⁵ Levene and Beatty: *Zeitschr. f. physiol. Chem.*, xlvii, p. 149, 1906.

ilar weight of moisture. This corresponds to approximately 3 molecules of water for 1 of alanine, the ratio, 1 alanine: 3 H_2O , requiring 3.99 per cent water.

Solubility of d-alanine phosphotungstate in varying concentrations of sulphuric acid.

Solutions each containing 0.250 gram of *d*-alanine, 5 grams of phosphotungstic acid, and varying amounts of sulphuric acid were made up to 10 cc. volume and left at 0° for forty-eight hours. The solutions were then decanted through dry filter papers into the 10 cc. burette of the aminometer (apparatus for determination of amino nitrogen) described in this *Journal*, xii, p. 275. The nitrogen in the measured volume of filtrate was determined by the nitrous acid method, and from the result the amount of alanine present in 100 cc. of filtrate was calculated. The percentages of sulphuric acid indicate grams per 100 cc. of solution.

TABLE I.

CONCENTRATION H_2SO_4	ALANINE IN 100 CC. OF FILTRATE
per cent	grams
3	0.56
4	0.38
5	0.36
6	0.30
8	0.19
10	0.14
10	0.15
12	0.16
14	0.18
16	0.18

As 0.250 gram of alanine combines with 3.5 grams of phosphotungstic acid, the excess of the latter in solution was 1.5 grams, or 15 grams per 100 cc. The above table indicates that, in the presence of this excess of phosphotungstic acid, sulphuric acid decreases the solubility of alanine phosphotungstate, the maximum effect of the sulphuric acid being exerted in 10 per cent concentration. Under these conditions the solubility of alanine at 0° is only 1 gram per 700 cc. of solution.

Effect of the concentration of free phosphotungstic acid on the solubility of d-alanine phosphotungstate in 10 per cent sulphuric acid at 0°.

Portions of 50 mgm. of *d*-alanine were dissolved in 5 cc. each of 20 per cent sulphuric acid in test tubes, and varying amounts of a solution containing 2 grams of phosphotungstic acid per cubic centimeter were added to the different solutions, all of which were then made up to 10 cc. with water allowed to stand thirty hours at 0°. The amounts of alanine remaining in solution were then determined as described in the preceding section. The excess phosphotungstic acid was estimated by subtracting from the amount added the 0.7 gram combining with 0.05 gram of alanine.

TABLE II.

PTA ADDED PER 100 CC.	EXCESS PTA PRESENT PER 100 CC.	ALANINE IN 100 CC. OF FILTRATE
<i>grams</i>		<i>grams</i>
15	8	0.22
20	13	0.21
25	18	0.18
30	23	0.15
40	33	0.14
60	53	0.15
80	73	0.13

It is evident that about 20 per cent of free, excess phosphotungstic acid in solution insures a maximum precipitation of the alanine at 0°. At 20°, in the presence of 20 per cent phosphotungstic acid solution, the solubility is 0.3 gram per 100 cc.

Time required for the precipitation of d-alanine phosphotungstate at 0°.

Portions of 0.05 gram of *d*-alanine were dissolved with 3 grams of phosphotungstic acid in 10 cc. of 10 per cent sulphuric acid. The solutions were left at 0° for varying periods, at the end of which they were decanted through dry filters, as in the solubility determinations described in the preceding sections, and the nitrogen remaining in solution was determined.

TABLE III.

TIME ALLOWED FOR PRECIPITATION	ALANINE IN 100 CC. FILTRATE
<i>hours</i>	<i>grams</i>
3	0.21
6	0.20
15	0.17
22	0.16
40	0.14

While the greater part of the alanine is precipitated in three hours, over twenty are required for the complete attainment of solubility equilibrium.

Solubility of dl-alanine in 10 per cent sulphuric acid containing varying concentrations of phosphotungstic acid.

The results in the following table show that the phosphotungstate of *dl*-alanine is more than twice as soluble at 0° as that of *d*-alanine. The conditions of the solubility tests were the same as those of the foregoing experiment.

TABLE IV.

PTA ADDED PER 100 CC.	EXCESS PTA PRESENT PER 100 CC.	ALANINE IN 100 CC. FILTRATE
<i>grams</i>		<i>grams</i>
10	3	0.43
20	13	0.35
30	23	0.35
60	53	0.37
80	73	0.37

Solubility of d-valine phosphotungstate in varying concentrations of sulphuric acid at 0°.

Portions of 0.4 gram of valine were dissolved with 6 grams of phosphotungstic acid each in 5 cc. of 2, 4, 6, 8, 10, and 12 per cent sulphuric acid respectively. The amount of phosphotungstic acid was found by a separate experiment to be a sufficient excess to depress the solubility of the valine to its minimum. The solutions were cooled to 0° and kept at that temperature for three days. The solubilities of the valine were then determined as in

the similar experiments with alanine. The solution with only 2 per cent of sulphuric acid showed no precipitate. The others showed crystalline precipitates varying in bulk with the concentration of the sulphuric acid. The percentages of sulphuric acid indicate grams per 100 cc.

TABLE V.

H ₂ SO ₄	VALINE IN 100 CC. OF FILTRATE
<i>per cent</i>	<i>grams</i>
4	4.95
6	2.78
8	1.87
10	1.21
12	0.88

At 20° the solubility in 10 per cent sulphuric acid in the presence of an excess of phosphotungstic acid is 3.4 grams per 100 cc.

Solubility of valine and alanine in varying concentrations of acetone.

As stated before, acetone was found a better agent than methyl or ethyl alcohol for throwing valine out of solution in the presence of the small proportions of alanine that escape precipitation with the main crop of alanine phosphotungstate. To ascertain the optimum proportion of acetone to add to the water solution of valine in order to cause it to crystallize most completely without carrying down alanine also, the solubilities of the two amino-acids in varying concentrations of acetone were determined at 20°. Fifteen cubic centimeters of the solvent were in each case shaken two hours with an excess of amino-acid, and 10 cc. of the filtered solution evaporated in a weighed dish.

TABLE VI.

ACETONE	VALINE SOLUBLE IN 100 CC. AT 20°	ALANINE SOLUBLE IN 100 CC. AT 20°
<i>per cent</i>	<i>grams</i>	<i>grams</i>
100	0.008	0.002
90	0.028	0.012
80	0.164	0.097
66.7	0.560	0.402
50	1.290	1.315

The following table shows that when 80 per cent acetone, in the ratio of from 2 to 7 volumes, is added to 1 volume of water, the solvent power of the water for alanine and valine is reduced to a point which remains nearly the same, whether 2, 3, 4, 5, 6, or 7 volumes of the 80 per cent acetone are added. The decrease in solubility caused by increasing the percentage of acetone is approximately compensated by the increase in volume.

TABLE VII.

80 PER CENT ACETONE ADDED TO 100 CC. OF WATER	ACETONE IN THE MIXTURE	SOLUBILITY IN 100 CC. OF THE MIXTURE		AMINO-ACID DISSOLVED IN THE TOTAL VOLUME OF MIXTURE	
		Alanine	Valine	Alanine	Valine
cc.	per cent	grams	grams	grams	grams
200	53.3	1.08	1.16	3.24	3.48
300	60.0	0.71	0.85	2.84	3.40
400	64.0	0.52	0.67	2.60	3.35
500	66.7	0.40	0.56	2.40	3.36
600	68.6	0.35	0.48	2.45	3.36
700	70.0	0.31	0.43	2.48	3.44

The solubilities in the third column were graphically interpolated from those given in the preceding table.

Separation of a mixture of d-valine and d-alanine.

The following separation serves as an example of the application of the method.

One gram each of *d*-valine and *d*-alanine was dissolved in 35 cc. of hot 10 per cent sulphuric acid (prepared by diluting 10 grams of acid to 100 cc.) with 23 grams of purified phosphotungstic acid. The solution was allowed to stand till it had cooled to room temperature, and was then placed in a refrigerator at 0° for twenty-four hours. The crystals which had separated formed a solid layer about the walls and bottom of the flask. The supernatant liquid was decanted off, and the crystals were redissolved on the water bath with 35 cc. of fresh 10 per cent sulphuric acid. Eight grams of phosphotungstic acid were then dissolved in the hot solution, which was cooled and placed in the refrigerator for twenty-four hours as before. The mother liquors were again decanted off, and the crystals were quickly washed on a suction funnel with

several small portions of a solution containing 10 grams of sulphuric acid and 20 grams of phosphotungstic per 100 cc., the washing solution being at a temperature of 0°.

Alanine. The crystals were transferred as completely as possible with a spatula from the funnel to a Jena beaker. A small residue adhering to the funnel and filter paper was washed into the beaker with hot water, and the flask in which the crystals had formed was also washed out with hot water, in order to obtain a few crystals of alanine phosphotungstate which the previous washing had not removed to the funnel. Enough water was added to the alanine phosphotungstate to bring the volume to 75–100 cc., and the beaker was covered and heated on the water bath until the crystals were dissolved to a slightly turbid solution. The latter was transferred to a 150 cc. measuring flask and diluted to the mark. Two cubic centimeters of the solution used for determination of amino nitrogen in the micro-apparatus gave 3.37 cc. of nitrogen gas at 25°, 758 mm., indicating 0.1398 gram of nitrogen, or 0.889 gram of alanine in the entire solution. The remaining 148 cc. of solution were treated as described on pp. 108 and 109 to remove phosphotungstic and sulphuric acids. The alanine regained weighed 0.83 gram, and gave the following figures on analysis.

Substance, 0.1222 gram; ash, 0.0013 gram; substance, ash-free, 0.1209 gram; CO₂, 0.1777 gram; H₂O, 0.0855 gram.

Rotation in 20 per cent HCl: Substance, 0.1029 gram = 0.1016 ash-free; solution, 1.9060 grams; concentration, 5.33 per cent; sp. gr., 1.1; rotation in 1 dm. tube, +0.80°.

	Found:	Calculated for <i>d</i> -alanine:
C.....	40.10	40.40
H.....	7.92	7.92
[α] _D ^{20°}	+13.7° ±0.2°	+13.7°

It is evident that the precipitate consisted of pure alanine phosphotungstate. The correction for the solubility of alanine as phosphotungstate in 70 cc. of solution under the conditions of precipitation and recrystallization is $0.70 \times 0.15 = 0.105$ gram of alanine. Adding this to the 0.889 gram precipitated gives 0.994 gram of alanine found to be present out of the 1 gram originally added.

Valine. The filtrate and washings from the alanine phosphotungstate were diluted to 150 cc. and 2 cc. of the solution taken

for determination of amino nitrogen. The nitrogen obtained measured 3.28 cc. at 25°, 758 mm., indicating 0.1362 gram of nitrogen in the entire solution. This is equivalent to 1.001 gram valine besides the 0.105 gram of alanine which, according to the solubility of alanine phosphotungstate, should be present. The remaining 148 cc. of solution were freed from sulphuric and phosphotungstic acids with lead acetate and concentrated, first in vacuum, then in a Jena glass dish on the water bath, until valine began to crystallize at the surface. About 3 volumes of 80 per cent acetone were stirred into the hot solution, which was then transferred, with the aid of more 80 per cent acetone, to an Erlenmeyer flask. The flask was stoppered and the valine allowed to crystallize in the ice box. The crystals, washed with 80 per cent acetone, weighed 0.78 gram, and gave the following analytical figures.

Analysis: Substance, 0.1198 gram (no ash); CO₂, 0.2258 gram; H₂O, 0.1108 gram.

Rotation in 20 per cent HCl: Substance, 0.0811 gram; solution, 1.291 grams; concentration, 6.28 per cent; sp. gr., 1.1; rotation in 1 dm. tube, +1.78° ± 0.01°.

	Found:	Calculated for d-valine:
C.....	51.38	51.24
H.....	9.41	9.47
[α] _D ^{20°}	+25.8°	+25.9°

The filtrate from the above crop of valine was concentrated to dryness, taken up with 25 cc. of 10 per cent sulphuric acid, and the alanine precipitated with 6.5 grams of phosphotungstic acid. The precipitate was dissolved in hot water and the solution diluted to 50 cc. Two cubic centimeters gave 0.52 cc. of nitrogen, equivalent to 0.05 gram of alanine in the entire solution. This precipitation could have been made a little more complete if it had been performed in the same manner as the first, using only 7 or 8 cc. of solution instead of 25, and recrystallizing once. The filtrate from the alanine phosphotungstate was also brought to 50 cc. and 2 cc. taken for a determination of amino nitrogen, which yielded 1.89 cc. of gas at 24°, 766 mm., equivalent to 0.0266 gram of nitrogen in the entire solution. This indicates, besides the 0.04 gram of alanine soluble in the 25 cc. of solution from which it was precipitated, 0.17 gram of valine. When the solution had

been freed from mineral acids and the product crystallized from dilute acetone, 0.13 gram of analytically pure valine was obtained.

Analysis: Substance, 0.1040 gram; ash, 0.0011 gram; substance, ash-free, 0.1029 gram; CO₂, 0.1935 gram; H₂O, 0.0896 gram.

	Found:	Calculated for C ₆ H ₁₁ O ₂ N:
C.....	51.28	51.24
H.....	9.74	9.47

The total amount of analytically pure valine regained was 0.91 gram, or, making allowance for the portions of solution removed for nitrogen determination, 0.93 gram. The amount present, as calculated from the nitrogen content of the filtrate from the alanine, was 1.001 grams. The loss of 0.07 gram in isolation is due partly to loss in crystallization, partly to adsorption by the heavy lead precipitates formed when the mineral acids are removed, these precipitates always adsorbing a few per cent of the amino-acid present.

CONCLUSION.

d-Alanine combines with phosphotungstic acid* in the ratio of approximately 1:14 by weight, forming a crystalline salt. At 0°, in a solution containing, per 100 cc., 20 grams or more of phosphotungstic acid in excess of the amount combining with the alanine, and 10 grams of sulphuric acid, the solubility of alanine is only 0.15 gram per 100 cc. The solubility of *d*-valine under the same conditions is 1.21 grams per 100 cc. By alternate crystallization of valine as the free amino-acid and of alanine as the phosphotungstate, one can effect a practically quantitative separation of a mixture of the two amino-acids.

THE GASOMETRIC DETERMINATION OF ALIPHATIC AMINO NITROGEN IN MINUTE QUANTITIES.

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In a previous number of this *Journal*¹ we have described an improved apparatus for the determination of amino nitrogen by the nitrous acid reaction. Its chief advantages over the form originally described² by us lay in its ability to be used an indefinite number of times without separating any of the parts, and in the fact that it permitted all the shaking to be done by a motor. By merely reducing its size this form of apparatus can be given an accuracy which brings the determination within the class of micro-methods. The gas burette of the micro-apparatus holds 10 cc. The upper part, measuring the first 2 cc., is of only 4 mm. diameter, and is divided into $\frac{1}{50}$ cc. divisions. The remainder is wider, and is divided into twentieths. In order to keep the correction necessary for the reagents small, it is preferable that the amounts of the latter should be reduced in proportion to the volume of nitrogen obtained for measurement. The deaminizing bulb is, therefore, of only 11 to 12 cc. content, and the 10 cc. burette on the larger apparatus is replaced by one of 2 cc. capacity. Only 10

¹ This *Journal*, xii, p. 275, 1912.

² *Ibid.*, ix, p. 185, 1911. The apparatus is designed only for use with a motor. It can be obtained from Emil Greiner, 45 Cliff Street, New York, with motor for either direct or alternating street current, or from Robert Goetze, Leipzig.

The substance in the nitrite which gives the small amount of gas obtained on blank determinations we have never been able to identify or remove. As a matter of fact, while some brands of commercial grades of nitrite are entirely unsuitable, others give results as good as those obtained with the most high priced "reagent" or "zur Analyse" preparations. For the last two years we have used the ordinary grade supplied by the Powers-Weightman-Rosengarten Company with uniformly good results.

cc. of nitrite solution and 2.5 cc. of acetic acid are required for an analysis, and the correction for the reagents is 0.06 to 0.12 cc., according to the quality of the nitrite employed. The same size of modified Hempel pipette (see previous article) can be used for the small as for the large apparatus, and, because of the small amounts of nitric oxide absorbed, it lasts for an almost indefinite number of analyses without change of the permanganate solution. With the micro-apparatus the error need not be more than 0.005 mgm. of nitrogen when 2 cc. or less of gas are measured, or 0.01 mgm. when more is obtained. Consequently one can analyze one-fifth the amount of substance required for the larger apparatus without reducing the percentage accuracy.

The advantages of the micro-apparatus are: (1) It requires only 0.5 mgm. of amino nitrogen for an analysis accurate to within 1 per cent. (2) It uses up relatively small amounts of reagents. (3) Having shorter dimensions and being of equally thick glass, it is relatively stronger than the larger apparatus. In point of rapidity the little apparatus has, if anything, a slight advantage over the large one. On a warm day we have made as many as ten accurate analyses per hour with the former. The minimum time required for the quantitative evolution of the nitrogen of α -amino-acids in the thoroughly shaken apparatus is, at 15° to 20°, five to four minutes; at 20° to 25°, three minutes; at 25° to 30°, two and a half to two minutes. Because of its conveniences, we now use for physiological work the smaller apparatus almost exclusively.

Practically the only alteration from the mode of operation, already detailed in the previous description of the larger apparatus, is in the speeds at which the deaminizing bulb and the Hempel pipette are shaken. During the first stage of the analysis³ the deaminizing bulb should be shaken by the motor at very high rate of speed, about as fast as the eye can follow, or an unnecessary amount of time is lost in freeing the apparatus from air. This stage is also much accelerated by warming the nitrite solution to 30° before it is used, in case a low room temperature has reduced the temperature of the solutions below 20°. In the third stage⁴ when the nitric oxide is being absorbed by the permanganate, the

³ This *Journal*, xii, p. 279, 1912.

⁴ *Ibid.*, p. 280.

Hempel pipette should be shaken not faster than twice per second. Absorption is approximately as fast as when more vigorous shaking is used, and the latter is likely to break off from the residual gas small bubbles, which stick under the nearly horizontal upper side of the pipette and escape being drawn back into the gas burette for measurement.

Because of the small amount of nitrogen to be measured, it is especially necessary that in the first stage the removal of the air should be complete. This is assured by shaking the solution in the deaminizing bulb back each time, in this stage, until the bulb is two-thirds filled with nitric oxide.

One point in setting up the apparatus appears to require especial emphasis. The hook or wire loop from which the deaminizing bulb is suspended⁵ should be perfectly rigid and hold the capillary outlet tube tightly. Otherwise the rapid shaking which is advantageous becomes, instead of a smooth vibration, a rattle, disagreeable to the operator and dangerous to the apparatus. Binding the tube to the holder with a strip of rubber band is a satisfactory method of insuring a firmly held apparatus.

The entire apparatus can be cleaned most conveniently by filling the burettes and deaminizing bulb with dichromate-sulphuric acid mixture. When the apparatus is in daily use it is a good practice to let it stand regularly over night filled with the cleaning mixture.

Two points in which every apparatus should be tested, as soon as it is set up, are the accuracy of the burettes and the tightness of the stopcocks. The two burettes are calibrated by weighing the water which they deliver; and the cocks are tested for their ability to remain air-tight when subjected to the suction or pressure of a column of water a meter high.

For most work, the solutions for analysis can be measured off with sufficient accuracy in the 2 cc. burette on the side of the deaminizing vessel. When especially accurate results are desired, however, one uses an Ostwald pipette, calibrated to deliver 1 or 2 cc. within 0.001 or 0.002 cc. respectively, and washes the burette twice, with six or seven drops of water distributed about the entire inner walls of the burette for each washing.

⁵ See photograph, this *Journal*, xii, p. 277.

The following results were obtained on four successive analyses from solutions measured in this manner, and illustrate fairly the accuracy which one can attain with the method. For each analysis 2 cc. of a 1 per cent solution of Kahlbaum's leucine were used, the amount of leucine being therefore 20 mgms.

NO.	N GAS	TEMPERATURE	PRESSURE	N EVOLVED	CALCULATED	ERROR
	cc.	deg. C.	mm.	mgms.	mgms.	mgms.
1	3.75	20	762	2.140	2.138	+0.002
2	3.74	20	762	2.133	2.138	-0.005
3	3.74	20	762	2.133	2.138	-0.005
4	3.77	21	762	2.141	2.138	+0.003

The following results, obtained with solutions measured from the 2-cc. burette, indicate that, when the latter is clean and the delivery careful, it gives nearly as consistent results as a pipette. For each analysis 2 cc. of a $\frac{N}{10}$ solution of alanine were taken. The time allowed for the reaction was two and a half minutes.

NO.	N GAS	TEMPERATURE	PRESSURE	N EVOLVED	CALCULATED	ERROR
	cc.	deg. C.	mm.	mgms.	mgms.	mgms.
1	2.56	28	760	1.398	1.401	-0.003
2	2.58	28	760	1.409	1.401	+0.008
3	2.57	28	760	1.403	1.401	+0.002
4	2.57	28	760	1.403	1.401	+0.002

IMPROVED METHODS IN THE GASOMETRIC DETERMINATION OF FREE AND CONJUGATED AMINO-ACID NITROGEN IN THE URINE.

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Total amino-acid nitrogen.

Our original method for the determination of the total amino-acid nitrogen¹ (free plus conjugated), although it gave accurate results and has been used successfully by ourselves and others, required a somewhat cumbersome manipulation before the urines were ready for the final amino determination. After being acidified and heated in the autoclave to hydrolyze the urea, the ammonia was boiled off on a hot plate with lime, a process requiring careful watching for an hour or more, and rather offensive because of the odors evolved. The calcium sulphate and hydrate were then filtered, the filtration and washing requiring another hour. The washings, of about 500 cc. volume, were then concentrated on the water bath, which required two or more hours additional.

These manipulations have been greatly simplified by the ascertainment of the fact that one has merely to filter off the alkaline solution obtained after adding the lime, and concentrate the filtrate on the water bath to dryness, in order to drive off every trace of ammonia. This process dispenses entirely with the troublesome boiling off of the ammonia. The washing of the precipitate of calcium salts can also be avoided to advantage by making the mixture up to a definite volume before filtering, and taking an aliquot portion of the filtrate for the rest of the determination. All the operations are furthermore rendered more convenient by the use of the micro-apparatus for determining the

¹ Levene and Van Slyke: *This Journal*, xii, p. 301, 1912.

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amino nitrogen, which permits one to work with relatively small volumes of liquid, and yet have sufficient material for duplicates.

The present method is the following: 25 cc. of urine are mixed with 1 cc. of concentrated sulphuric acid and heated in an autoclave at 180° (oil bath temperature) for one and a half hours. The solution is then transferred to a 50-cc. flask and 2 grams of powdered calcium hydrate are added. The mixture is thoroughly shaken, made up to 50 cc., and filtered through a dry folded filter. Twenty cubic centimeters of the filtrate are measured into a Jena glass evaporating dish and concentrated to dryness on the water bath, the process of concentration requiring about a half hour. The residue is moistened with 1 cc. of 50 per cent acetic acid to bring the calcium hydrate and carbonate into solution, and is then washed into a 10 cc. flask and filled up to the mark. One can either use the entire solution for determination of the amino nitrogen in the large amino apparatus, or use 2-cc. portions for the micro-apparatus.

The length of time which the nitrous acid solution should be shaken in order to drive off all the amino nitrogen depends somewhat on the temperature. When the latter is $15-20^{\circ}$ the time should be five to four minutes; for $20-25^{\circ}$ it is three minutes; for $25-30^{\circ}$, two and a half to two minutes. It is preferable that the solution should be shaken vigorously with a motor and the time kept down to these limits, for the sake not only of rapidity but of accuracy. The reason for this is, that, even after removal of the ammonia and urea, urines contain small amounts of substances which belong to the class of *slowly* reacting amines, and are therefore not α -amino-acids. The correction for this nitrogen can be ascertained in the same manner as the urea correction in amino determination on the blood,² by continuing the reaction, after the gas from the amino-acids has all been driven off, for a length of time equal to that utilized in decomposing the amino-acids (two to five minutes, according to the temperature), and then measuring the nitrogen that has been evolved during this second reaction-period. The correction is so small and constant, however, amounting to 0.2-0.3 per cent of the urine nitrogen, that it will for most work be found unnecessary to take it into account.

² Van Slyke and Meyer: *This Journal*, xii, p. 402, 1912.

The following results were obtained with normal human urines. For the final determination, 2 cc. of solution, equivalent to 2 cc. of urine, were used in the micro-apparatus. The temperature was 25°, the pressure 758 mm. for all measurements.

TABLE I.
Total amino nitrogen (free and conjugated).

NO.	TOTAL N PER 100 CC.	N GAS	CORRECTED FOR AMINES OTHER THAN AMINO-ACIDS	AMINO NITROGEN PER 100 CC. URINE		PER CENT OF TOTAL N IN FORM OF AMINO-ACID NH_2	
				Uncor- rected	Corrected (Amino- acids only)	Uncor- rected	Corrected
	<i>grams</i>	<i>cc.</i>		<i>mgms.</i>	<i>mgms.</i>		
1	1.211	1.15	1.05	31.1	28.4	2.57	2.35
		1.18	1.04	31.9	28.1	2.63	2.32
2	1.750	1.38	1.24	37.5	33.7	2.14	1.93
		1.30	1.26	37.7	34.3	2.15	1.96
3	0.833	1.19	1.08	32.4	29.3	3.89	3.52
		1.19	1.06	32.4	29.8	3.89	3.46
4	1.747	1.32	1.15	36.0	31.4	2.06	1.80
		1.30	1.15	35.3	31.4	2.02	1.80
5	1.309	1.10	0.97	31.0	27.0	2.37	2.09
		1.10	0.95	31.0	26.5	2.37	2.04

Free amino-acid nitrogen.

At the time our first paper was published we had been unable to find an agent which would remove or destroy the urea without either hydrolyzing conjugated amino-acids (hippuric acid, peptone, etc.) or removing free ones. Treatment in an autoclave, as described in the first part of this paper, efficiently destroys the urea, but it also hydrolyzes the conjugated amino-acids. Mercuric acetate with alkali precipitates urea completely, but it also precipitates almost all of the amino-acids. We were therefore forced to take advantage of the fact that urea reacts to the extent of only about 3 per cent with nitrous acid in the time that amino-acids react with 100 per cent of their nitrogen. After the amino-acid nitrogen has been driven off and measured one can ascertain

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the rate at which urea is evolving nitrogen in the same mixture, and thus make a correction for the small percentage of urea nitrogen decomposed while the amino-acids were finishing the reaction. The method is satisfactory when, as in normal blood, the excess of urea is not too great, but in the urine the urea nitrogen is about 100 times the normal free amino-acid nitrogen. For this reason the method could not be depended upon to give results more accurate than ± 0.5 per cent of the total nitrogen of the urine, and was therefore of value to determine amino-acids only when they were present in abnormally large amounts.

Recently, however, Marshall³ has found in the urease of the soy bean the specific reagent for the destruction of urea. He shows that the water extract of the beans (prepared by extracting the pulverized beans with 10 parts of water for an hour at room temperature, then warming the mixture to 35° , adding one-tenth volume of $\frac{N}{10}$ HCl to coagulate proteins, and filtering) completely hydrolyzes urea in the space of a few hours at 35° to ammonium carbonate. We have been able to confirm his results, and find furthermore that the extract under the conditions used does not appreciably hydrolyze hippuric acid, casein, or peptone, nor deaminate amino-acids.

One peculiarity which we have noticed is that the enzyme does not appear to follow the law of mass action. A given amount is required to decompose a urine under given conditions, and the dilution of the reacting substances can be varied greatly without much affecting results. It is essential, therefore, that the amount of extract taken should be sufficient to completely decompose all the urea present. This is most certainly assured by testing measured portions of the extract with urines of concentration at least as great as that of those to be analyzed, and ascertaining the proportion of enzyme necessary to give a maximum amount of ammonia. Our method is to take 3-cc. portions of urine in 100 cc. test tubes, add 1.0-, 1.5-, 2.0-, 2.5-, and 3.0-cc. portions of extract with a few drops of toluol to the respective tubes of the series, and place in a bath at 35° for three hours, or sixteen to twenty hours at room temperature. To each tube 2 cc. of saturated potassium carbonate solution are then added, and the

³ Marshall: *This Journal*, xiv, p. 283, 1913.

ammonia is driven into 25 cc. of $\frac{N}{10}$ hydrochloric acid by ten minutes' aeration according to Folin's recent method.⁴ As an example the amounts of $\frac{N}{10}$ acid neutralized in one such test were 17.85, 19.50, 20.20, 20.05, and 20.20 cc. respectively. Under the conditions, 3 cc. of urine required 2 cc. of enzyme solution.

The soy bean extract was tested for proteolytic activity in the following experiment. The extract added to 2 per cent urea solution in the proportions of 2 volumes of extract to 5 of urea solution, completely decomposed the latter in sixteen hours at room temperature. To test the action on a protein the following solution was prepared: 25 cc. H_2O ; 10 cc. soy bean extract; 0.125 gram casein; 1.0 cc. 0.1 N NaOH (to neutralize the casein); 0.2 gram NaCl.

In this and subsequent experiments toluene was used as preservative. The solution was alkaline to alizarine, acid to litmus. Two-cubic-centimeter portions, taken at once and after the solution had stood sixteen hours at 25°, were analyzed in the micro-apparatus for amino nitrogen. The time of reaction was four minutes in each case. The results were:

At once.....0.26 cc. N_2 at 21°, 767 mm.

After sixteen hours.....0.29 cc. N_2 at 19°, 767 mm.

A control performed on a solution without casein, but otherwise like the above, gave 0.20 cc. nitrogen gas under the same conditions. The free amino nitrogen in the amount of casein present in the first solution (5.5 per cent of the total nitrogen, see later paper by Van Slyke and Birchard) would yield 0.09 cc. of nitrogen gas if given time to react completely. It is evident from the above that, under conditions that result in complete decomposition of urea, casein is not appreciably hydrolyzed by the urease. In fact a solution of sodium caseinate without enzyme showed under the same conditions as the result of autohydrolysis, more increase in amino nitrogen (0.05 cc.) than that noted above.

In order to test the extract for the presence of an erepsin, the following solution was prepared: 25 cc. H_2O ; 10 cc. soy bean extract; 0.200 gram Siegfried's peptone from fibrin; 0.200 gram NaCl.

⁴ This *Journal*, xi, p. 507, 1912.

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The solution was slightly alkaline to alizarine, acid to litmus. Two-cubic-centimeter portions were taken for amino nitrogen determinations. The results of the determinations are given in the following table.

TABLE II.

PERIOD OF ACTION	N ₂ GAS	TEMPERATURE	PRESSURE
<i>hours</i>	<i>cc.</i>	<i>deg. C.</i>	<i>mm.</i>
0	0.66	22	768
3	0.69	22	768
24	0.70	20	766
72	0.82	26	766

Within the period required to decompose urea (sixteen hours) the action of the extract on the peptone is barely discernible.

To test the effect of the enzyme on amino-acids, the artificially digested meat termed "ereptone" and manufactured at Hoechst a. M. according to Abderhalden's method was used as a substrate. The decomposition of the original proteins into amino-acids was almost complete in the preparation used, as heating for twenty-four hours at 100° with 20 per cent hydrochloric acid increased the amino nitrogen from 63 per cent only up to 67 per cent of the total nitrogen. The use of such a preparation, which probably contains all of the amino-acids found in the body, and in about the proportions in which they exist in the body as a whole, affords a more practical test for the purpose of the experiment than would the utilization of some of the individual amino-acids. The solution contained: 25 cc. H₂O; 10 cc. soy bean extract; 0.200 gram ereptone (containing 12.8 per cent N); 0.200 gram NaCl; 0.5 cc. 0.1 N NaOH to render the solution just alkaline to alizarine. Amino determination on 2-cc. portions gave:

At once.....1.60 cc. N₂ at 19°, 767 mm.

After sixteen hours.....1.59 cc. N₂ at 19°, 771 mm.

No deamination whatever occurred.

To test the extract for its ability to hydrolyze hippuric acid the following solution was prepared: Kahlbaum's hippuric acid, 0.200 gram; 0.1 N NaOH (1 equivalent), 11.1 cc; H₂O, 10 cc; soy bean extract, 10 cc.

The solution was acid to litmus, alkaline to Congo and alizarine. Two-cubic-centimeter portions were taken for analysis. The results were:

At once.....	0.20 cc. N ₂ at 21°, 764 mm.
After sixteen hours.....	0.24 cc. N ₂ at 21°, 764 mm.

The increase of 0.04 cc. indicates the hydrolysis of 2 per cent of the hippuric acid present. Whether this was due to the action of the extract or to spontaneous splitting of the hippuric acid was not determined. The effect is, in any case, negligible so far as its influence on urine analyses is concerned.

All the above experiments were repeated, with similar results. They show that the soy bean extract, under the conditions used for complete decomposition of urea, does not hydrolyze casein, nor, to a significant extent, peptone or hippuric acid, nor does it deaminate amino-acids.

Method for free amino-acid nitrogen.

The proportion of extract necessary to completely hydrolyze urines of the maximum concentration is determined as described on p. 128. To 25 cc. of urine in a 50-cc. flask one adds the required amount of extract (usually about 15 cc. with the beans which we used) and lets the mixture stand for about one and a half times the interval which has been found sufficient to effect the maximum decomposition of urea, as observed by titration of the ammonia. These conditions assure decomposition of the last traces of urea. At the end of the digestion period 10 cc. of a 10 per cent suspension of calcium hydrate are added, and the mixture is shaken and diluted up to the 50 cc. mark. It is then filtered through a dry folded filter, and 20 cc. of the alkaline filtrate are concentrated in a Jena glass dish to dryness on the water bath, this process driving off all the ammonia (hippuric acid is not appreciably affected by this treatment). The residue is moistened with 1 cc. of 50 per cent acetic acid, washed into a 10-cc. measuring flask, and diluted to the mark. One uses the entire solution for a determination in the larger amino apparatus, or 2 cc. for duplicates in the smaller. The reaction period with the nitrous acid should be kept as short as possible for the reasons given on p. 126, and the

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correction for the amines other than amino-acids can be made in the same manner.

The results tabulated below were obtained with the same urines used for determination of total amino nitrogen. The amount of enzyme used was 15 cc.; the time allowed for it to act, five hours at 36°, three hours having been the period in which the proportion of extract used gave a maximum yield of ammonia in a previous test.

TABLE III.

Free amino nitrogen. All determinations at 26°, 760 mm. Reaction period two and a half minutes.

NO.	TOTAL N PER 100 CC. URINE	N GAS	CORRECTED FOR AMINES OTHER THAN AMINO-ACIDS	AMINO N PER 100 CC. URINE		PER CENT OF TOTAL N IN FORM OF AMINO-ACID NH ₂	
				Uncor- rected	Corrected (Amino-acid N only)	Uncor- rected	Corrected
	grams	cc.		mgms.	mgms.		
1	1.211	0.36	0.28	9.7	7.5	0.80	0.62
		0.34	0.26	9.2	7.2	0.76	0.58
2	1.750	0.48	0.35	13.0	9.8	0.74	0.56
		0.48	0.35	13.0	9.8	0.74	0.56
3	0.833	0.31	0.25	7.7	6.8	0.93	0.82
		0.31	0.25	7.7	6.8	0.93	0.82
4	1.747	0.55	0.33	15.0	8.9	0.85	0.51
		0.54	0.33	15.0	8.9	0.83	0.51
5	1.309	0.28	0.18	8.0	5.0	0.61	0.38
		0.28	0.18	8.0	5.0	0.61	0.38

The control solution, containing 15 cc. of extract with 25 cc. of water in place of the urine, gave 0.20 cc. of nitrogen gas during the first two and a half minutes of the reaction at 26°, and 0.05 cc. during the second two and a half minutes. These amounts, which were found without appreciable deviation in several duplicates, were subtracted from the volumes of gas read at each determination. The corrections for the volume of gas evolved by amines other than amino-acids were made as described on p. 126. Duplicate amino determinations with 2-cc. portions were made in each case with the micro-apparatus.

It will be noted that the amount of nitrogen evolved by amines other than amino-acids is practically the same, amounting to 0.2–0.3 per cent of the total nitrogen of the urine, whether the urea was removed by hydrolysis with sulphuric acid in the autoclave, or by the urease. When this correction is determined, we believe that the methods for both free and total amino nitrogen described above give with a close degree of approximation the actual amount of amino nitrogen present in the form of amino-acids. As the correction is relatively small and constant, it is probable that in most work, where comparative results chiefly are desired, it will be unnecessary to take it into account.

Hippuric acid.

Henriques and Sørensen have ascertained the conditions for the complete extraction of hippuric acid from urine with ethyl acetate, its subsequent hydrolysis to glycocoll and benzoic acid with hydrochloric acid, and its determination by titration of the amino nitrogen of the glycocoll by the formol method.⁵ The same methods can be applied, making the final determination by the gasometric method instead of the formol titration. When the gasometric method is applied, the results must be multiplied by the factor 0.93, as glycocoll, unlike the other amino-acids, gives off several per cent more gas than the volume corresponding to its nitrogen content. Using this factor, however, one can obtain without trouble results accurate to within 1 per cent of the total glycocoll determined. The gasometric method has some advantages over the formol method,⁶ and in this case should be particularly convenient because it simplifies the process of extraction by permitting one to work with small volumes of urine. As work from Henriques and Sørensen hardly requires confirmation of its reliability, and the substitution of the gasometric for the formol method in the final determination is an obvious modification, a more detailed discussion here appears unnecessary.

⁵ *Zeitschr. f. physiol. Chem.*, lxiii, p. 27, 1910; lxiv, p. 120, 1911.

⁶ *This Journal*, xii, p. 302, 1912.

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CONCLUSION.

The previously published process of determining the total amino-acid nitrogen (free amino-acids+conjugated amino-acids in the form of hippuric acid, peptides, proteins, etc.) has been simplified so that the operation is much shortened and the more laborious parts, boiling off ammonia and washing bulky precipitates, are dispensed with. The free amino-acids alone can readily be determined after decomposition of the urea with soy bean urease, which hydrolyzes urea completely without either freeing conjugated amino-acids or deaminizing free ones. The applicability of the gasometric method for the determination of hippuric acid is indicated.

RESEARCHES ON PURINES. XIII.¹

ON 2,8-DIOXY-1,6-DIMETHYLPURINE AND 2,6-DIOXY-3,4-DIMETHYL-5-NITROPYRIMIDINE (α -DIMETHYLNITROURACIL).²

BY CARL O. JOHNS AND EMIL J. BAUMANN.

(From the Sheffield Laboratory of Yale University.)

(Received for publication, August 26, 1913.)

We find that an aqueous solution of the sodium salt of 2-oxy-4-methyl-5-nitro-6-aminopyrimidine³ (I) reacts readily with dimethylsulphate and gives an 80 per cent yield of the corresponding dimethyl derivative. It seemed probable that the compound thus formed was 2-oxy-3,4-dimethyl-5-nitro-6-aminopyrimidine (II), because, in our previous work on alkylations of pyrimidines which contained oxygen in position 2 and an amino or alkylamino group in position 6 we found that the alkyl group entered position 3 in the pyrimidine ring.⁴ The following experiments show conclusively that in the case now under consideration the alkyl group also entered position 3.

The substance obtained by methylating 2-oxy-4-methyl-5-nitro-6-aminopyrimidine was heated with 25 per cent sulphuric acid under pressure. This treatment removed the amino group and a good yield of a 2,6-dioxy-5-nitro-dimethyl-pyrimidine was obtained. Two such compounds can exist in which one of the methyl groups is attached to nitrogen in the urea grouping of the pyrimidine ring and the other methyl group attached to the carbon atom in position 4, namely, 2,6-dioxy-1,4-dimethyl-5-nitropyrimidine (VII) and 2,6-dioxy-3,4-dimethyl-5-nitropyrimidine (III). Lehman⁵ ob-

¹ Johns and Baumann: this *Journal*, xv, p. 515, 1913. The present investigation was aided by a grant from the Bache fund.

² Behrend and Dietrich: *Ann. d. Chem.* (Liebig), cccix, p. 266, 1899; Behrend and Thurm: *ibid.*, ccexxiii, p. 163, 1902.

³ Johns: *Amer. Chem. Journ.*, xli, p. 60, 1909.

⁴ Johns: this *Journal*, xi, p. 75, 1912; xiv, p. 3, 1913.

⁵ Lehman: *Ann. d. Chem.* (Liebig), ccliii, p. 84, 1899.

tained one of the above compounds by the action of methyl iodide on the potassium salt of nitromethyluracil.⁶ His compound melted at 149°C. and the structure assigned to it was 2,6-dioxy-1,4-dimethyl-5-nitropyrimidine⁷ (VII). Our 2,6-dioxy-5-nitro-dimethylpyrimidine melts at 191°C. Hence, it cannot be identical with the compound obtained by Lehman and if the correct structure has been assigned to his compound ours must be 2,6-dioxy-3,4-dimethyl-5-nitropyrimidine (III).

Behrend and Köhler⁸ have shown that fuming nitric acid not only nitrates 4-methyluracil (X) but also oxidizes the methyl group forming 2,6-dioxy-4-carboxyl-5-nitropyrimidine (XI) and that this latter compound loses carbon dioxide with the consequent formation of 2,6-dioxy-5-nitropyrimidine or nitrouracil (XII).

We found that 2,6-dioxy-5-nitro-dimethylpyrimidine was also oxidized by fuming nitric acid and that the 2,6-dioxy-3-methyl-4-carboxyl-5-nitropyrimidine (VI) lost carbon dioxide which resulted in the formation of 2,6-dioxy-3-methyl-5-nitropyrimidine (IX). The structure of this compound has been firmly established by the work of Behrend and his collaborators.⁹ It melts at 255°C. and contains one molecule of water of crystallization and is therefore readily distinguished from its isomer 2,6-dioxy-1-methyl-5-nitropyrimidine¹⁰ (VIII) which melts at 263°C. and does not contain water of crystallization. The compound obtained by us contained water of crystallization and melted at 255°C. It was therefore 2,6-dioxy-3-methyl-5-nitropyrimidine. Hence in methylating 2-oxy-4-methyl-5-nitro-6-aminopyrimidine the methyl group entered position 3 and the compound formed was 2-oxy-3,4-dimethyl-5-nitro-6-aminopyrimidine (II).

The latter compound was reduced rapidly by the action of freshly precipitated ferrous hydroxide but the reaction was not smooth. After isolating about 40 per cent of the calculated weight of 2-oxy-3,4-dimethyl-5,6-diaminopyrimidine (V) a tarry by-product remained.

⁶ Behrend: *Ann. d. Chem. (Liebig)*, cexl, p. 3, 1887.

⁷ *Beilstein's Handbuch*, i, p. 1350 (third edition).

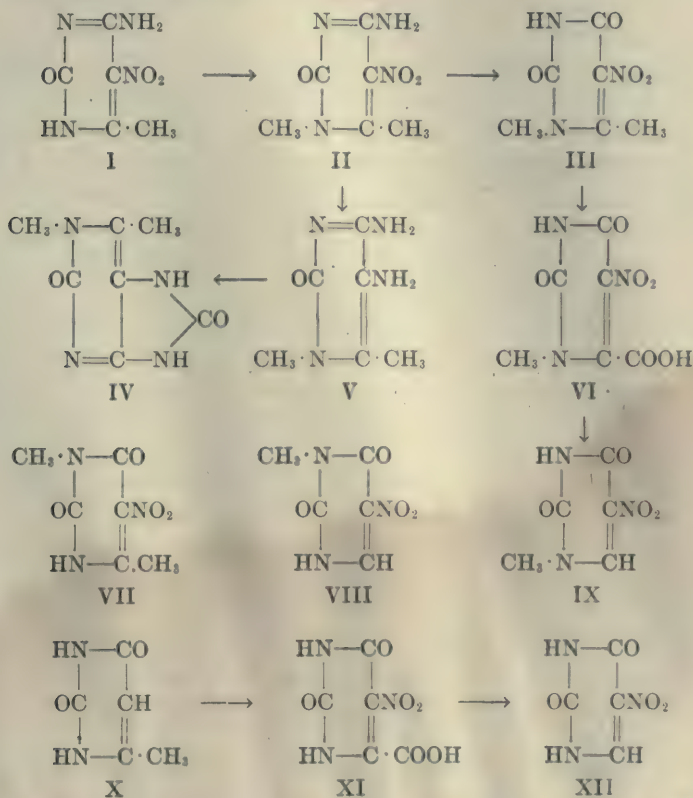
⁸ Behrend: *Ann. d. Chem. (Liebig)*, cexxix, p. 32, 1885; Köhler: *ibid.*, ccxxxv, p. 50, 1886.

⁹ Behrend and Thurm: *Ann. d. Chem. (Liebig)*, ccxxiii, p. 163, 1902.

¹⁰ *Ibid.*

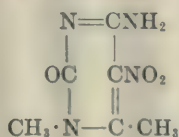
When 2-oxy-3,4-dimethyl-5,6-diaminopyrimidine was heated with urea we obtained an excellent yield of 2,8-dioxy-1,6-dimethyl-purine (IV).

These researches will be continued.



EXPERIMENTAL PART.

2-Oxy-3,4-dimethyl-5-nitro-6-aminopyrimidine.



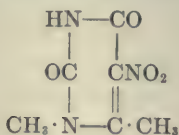
Ten grams of pulverized 2-oxy-4-methyl-5-nitro-6-aminopyrimidine¹¹ were dissolved in 100 cc. of hot water containing 2.8 grams of sodium hydroxide. After cooling this solution to room temperature, 10 grams of dimethylsulphate were added and the mixture was shaken to keep the dimethylsulphate in suspension. In less than five minutes crystals began to form. The mixture was shaken two or three minutes longer and then allowed to stand until it gave an acid reaction, which usually required less than fifteen minutes. Heat was evolved during the reaction and, after cooling, the precipitate was filtered off and washed with a little cold water and alcohol. The yield was 8.7 grams or 80 per cent of theory. This substance contained but a trace of the original 2-oxy-4-methyl-5-nitro-6-aminopyrimidine and was pure enough for subsequent experiments. The 2-oxy-3,4-dimethyl-5-nitro-6-aminopyrimidine dissolved readily on continued boiling in water and on cooling the solution slowly it crystallized in lustrous prisms that formed radiating clusters or sheaves. These crystals did not have a definite melting point but began to darken at about 170°C. and effervesced at 190° to 195°C. They were moderately soluble in hot alcohol, slightly soluble in boiling benzene and insoluble in ether. They dissolved readily in dilute hydrochloric acid and glacial acetic acid. They formed yellow solutions in strong alkalies and were moderately soluble in ammonium hydroxide. The crystals which were obtained from aqueous solutions possessed a pearly luster. This was lost on drying over sulphuric acid in a desiccator for one or two days though analyses showed that one-half molecule of water of crystallization still remained.

I. 2.0855 grams of substance dried over sulphuric acid for twenty-four hours lost 0.0093 gram at 120°-130°C.

II. 2.8975 grams of substance dried over sulphuric acid for 48 hours lost 0.1375 gram at 120°-130°C.

	Calculated for $C_6H_8O_3N_4 \cdot \frac{1}{2}H_2O$:	Found:	
		I	II
H_2O	4.66	4.76	4.73
	Calculated for $C_6H_8O_3N_4$:	Found:	
		I	II
N.....	30.43	30.38	30.54

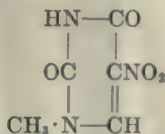
¹¹ Johns: *loc. cit.*

2,6-Dioxy-3,4-dimethyl-5-nitropyrimidine.

Three grams of 2-oxy-3,4-dimethyl-5-nitro-6-aminopyrimidine from the above experiment were dissolved in 20 cc. of 25 per cent sulphuric acid and the solution was heated in a sealed tube at 160°C. for two hours. When the contents of the tube were cooled a deposit of long, slender prisms was obtained. A second crop was isolated by neutralizing the filtrate with barium hydroxide, filtering, and concentrating this filtrate. The yield was 75 per cent of theory. The crude substance melted at 186° to 190°C. When recrystallized from alcohol the melting point was 191°C. It was easily soluble in hot water and on cooling the solution it crystallized rapidly in slender prisms. It also dissolved readily in hot alcohol, slightly in benzene but did not dissolve in ether. Dilute alkalis dissolved it easily.

	Calculated for $\text{C}_6\text{H}_7\text{O}_4\text{N}_3$:	Found:	
		I	II
N.....	22.70	22.61	22.62

The oxidation of 2,6-dioxy-3,4-dimethyl-5-nitropyrimidine with nitric acid. The formation of 2,6-dioxy-3-methyl-5-nitropyrimidine.¹²



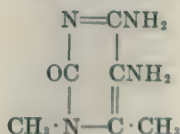
One gram of 2,6-dioxy-3,4-dimethyl-5-nitropyrimidine was dissolved in 10 cc. of nitric acid of specific gravity 1.5 and 2 cc. of concentrated sulphuric acid were added. The solution was heated on the water bath for one and one-half hours. Oxidation took place with effervescence and the liberation of brown fumes. The solution was diluted with water and the acids were neutralized with ammonia. On evaporating to dryness and washing with cold

¹² Behrend and Thurm: *Ann. d. Chem.* (Liebig), cccxxiii, p. 164, 1902.

water to remove salts, there remained a crystalline substance that weighed 0.2 gram. This melted at 254° to 255°C. and when recrystallized from water it melted sharply at 255° to 256°C. The crystals contained water of crystallization and when mixed with a pure sample of 2,6-dioxy-3-methyl-5-nitropyrimidine the melting point remained the same. Hence, the methyl group attached to nitrogen was in position 3 in the pyrimidine ring. The substance was dried at 120° to 130°C.

	Calculated for $C_6H_8O_4N_4$:	Found:
N.....	24.56	24.44

2-Oxy-3,4-dimethyl-5,6-diaminopyrimidine.

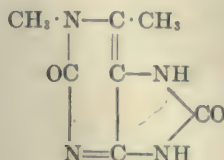


Five grams of 2-oxy-3,4-dimethyl-5-nitro-6-aminopyrimidine were dissolved in a mixture of 50 cc. of concentrated ammonia and 75 cc. of water by warming gently. This solution was cooled to room temperature and a hot, concentrated aqueous solution of 53 grams of crystallized ferrous sulphate was added gradually. Reduction took place rapidly and was accompanied with the liberation of heat. A solution of 63 grams of barium hydroxide was added to precipitate the sulphate and the excess of baryta was removed by adding ammonium carbonate. The mixture was well shaken and filtered after standing for an hour. The filtrate was evaporated to a small volume on the water bath and then cooled. A dark crystalline mass separated. This was dissolved in hot water and decolorized with blood coal. The diaminopyrimidine was thus obtained pure and colorless in the form of burrs that consisted of small plates. The yield was about 40 per cent of theory. The crystals possessed a pearly luster. They were very soluble in hot water and crystallized well on cooling the solution. They were moderately soluble in boiling alcohol but did not dissolve in benzene or ether. Dilute acids dissolved them easily. They did not exhibit a definite melting point but began to decompose at about 230°C. When an aqueous

solution of this substance was added to a cold ammoniacal silver solution a white gelatinous precipitate resulted and, on heating, a silver mirror was produced.

	Calculated for $C_6H_{10}ON_4$:	Found:
N.....	36.36	36.37

2,8-Dioxy-1,6-dimethylpurine.



Two grams of 2-oxy-3,4-dimethyl-5,6-diaminopyrimidine and 2 grams of urea were pulverized together and the mixture was heated for an hour at 170° to 180°C. in an oil bath. The mass melted to a liquid and frothing occurred while there was a copious evolution of ammonia. After some twenty-five minutes the reaction subsided and the mixture became a solid mass. After cooling, the reaction-product was dissolved in dilute ammonia and the solution was clarified with blood coal. After boiling off most of the ammonia, the solution was acidified with acetic acid whereupon crystals of the purine began to separate as the solution cooled. After two hours the crystals were filtered off. Another crop was obtained by concentrating the filtrate. The yield was 2 grams or 85 per cent of the calculated weight. The portion used for analysis was recrystallized from water. This purine dissolves in about 60 parts of boiling water and is slightly soluble in hot alcohol but does not dissolve in benzene or ether. It dissolves readily in hydrochloric acid or alkalis. It crystallizes from water in burrs composed of small prisms that contain one molecule of water of crystallization. It decomposed at 260° to 265°C. It did not form a difficultly soluble picrate or barium salt. Its water solution gave a gelatinous precipitate with mercuric chloride. This was soluble in hot water but reappeared on cooling the solution. With an ammoniacal silver solution a white precipitate was obtained. This did not darken when the contents of the test tube were boiled. Nitric acid oxi-

dized the purine readily and on careful evaporation a yellow crust remained. This became rose colored when treated with alkalies.

0.8798 gram of substance lost 0.0825 gram at 120° to 130°C.

	Calculated for	Found:
	$C_7H_8O_2N_4 \cdot H_2O$:	
H_2O	9.09	9.29
	Calculated for	Found:
	$C_7H_8O_2N_4$:	
N.....	31.11	31.32

POLYATOMIC ALCOHOLS AS SOURCES OF CARBON FOR LOWER FUNGI.

By RAY E. NEIDIG.

(From the Chemical Section of the Iowa Agricultural Experiment Station.)

(Received for publication, August 30, 1913.)

The carbon nutrition of the lower fungi has been studied quite extensively. The diversity of simple organic substances which molds are able to utilize as sources of carbon is indeed surprising. Not only do the naturally-occurring sugars supply the carbon requirements of these fungi, but many other substances, among them laboratory products not known to occur in nature, appear to be more or less readily utilized by these organisms. The vigor of the culture, however, may vary considerably with the nature of the substrate. Many substances on which only a scant growth can be obtained have been reported in the literature as available sources of carbon. It is of course impossible to express availability of a particular substrate for a given organism on an adequate quantitative basis, yet some distinction should be made between a sparse growth and a vigorous culture.

The cultures herein described were made for the purpose of determining differences in availability in the series of polyatomic alcohols. The substances selected represent a series differing progressively by the group CHOH . They all occur in nature, the first three in the form of esters, the others in the free state. Isomeric synthetic products will not be considered here. The series represents therefore substances containing one to six alcohol radicals and a similar number of carbon atoms, and may be designated by the general formula $\text{C}_n\text{H}_{2n+2}\text{O}_n$. Following is the list:¹

NUMBER OF CARBON ATOMS AND ALCOHOL RADICALS	SUBSTANCE	NUMBER OF CARBON ATOMS AND ALCOHOL RADICALS	SUBSTANCE
1	Methyl alcohol	5	Adonitol
2	Ethylene glycol	6	Mannitol
3	Glycerol	6	Dulcitol
4	Erythritol	6	Sorbitol

¹Several of these substances have been reported by Emmerling (*Centralbl. f. Bakt.*, x, II, p. 273) as available for *Aspergillus niger*, but quantitative differences were not considered.

These substances were introduced into Czapek's medium in place of the usual sugar. No other source of carbon was present. Inoculation was made with the spores of pure cultures, and the tubes were allowed to remain in the dark at room temperature. The cultures were examined at the end of the first, second and third week. The following notation will be used to designate the appearance of the culture:

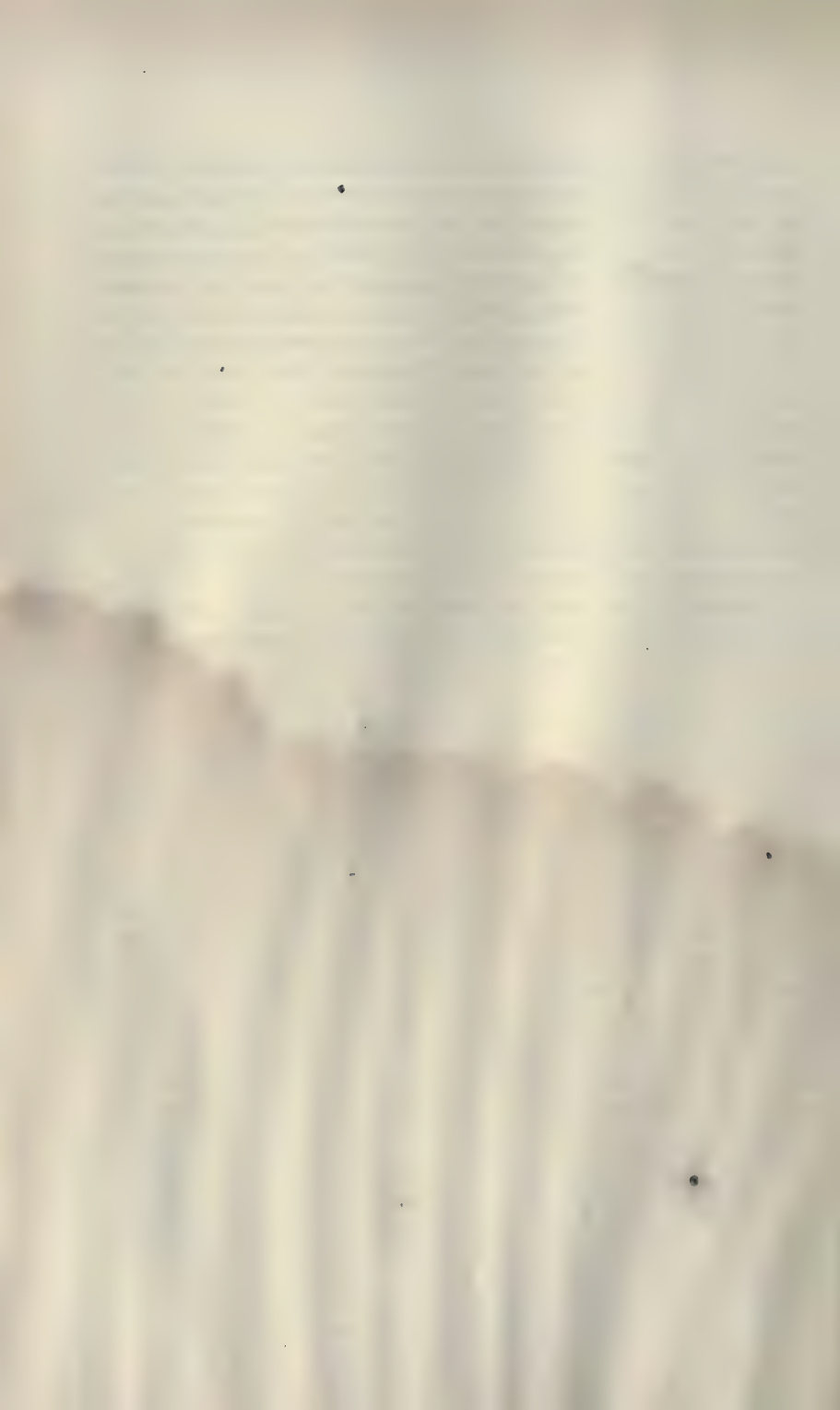
+++.....good normal culture
 ++.....medium growth
 +.....slight growth
 G.....germination or submerged hyphae only
 O.....no growth

ORGANISM	AGE OF CULTURE IN WEEKS	SOURCE OF CARBON									
		None	Methyl alcohol	Ethylene glycol	Glycerol	Erythritol	Adonitol	Sorbitol	Dulcitol	Mannitol	Sucrose
<i>Aspergillus niger</i>	1	O	O	O	+++	+	G	+++	+++	+++	+++
	2	O	O	G	+++	+	G	+++	+++	+++	+++
	3	O	O	G	+++	+	G	+++	+++	+++	+++
<i>Aspergillus clavatus</i>	1	O	O	G	+++	+	+	+	G	+++	+++
	2	O	O	G	+++	+	+	+	G	+++	+++
	3	O	O	G	+++	+	+	++	G	+++	+++
<i>Aspergillus fumigatus</i>	1	O	O	G	+++	+	+	+++	+++	++	+++
	2	O	O	+	+++	+++	++	+++	+++	+++	+++
	3	O	O	+	+++	+++	++	+++	+++	+++	+++
<i>Penicillium expansum</i>	1	O	O	G	+++	+	+	+++	+++	++	+++
	2	O	O	+	+++	+++	++	+++	+++	+++	+++
	3	O	O	+	+++	+++	++	+++	+++	+++	+++
<i>Fusarium oxysporium</i>	1	O	O	+	+++	G	+++	+++	+++	+++	+++
	2	O	O	++	+++	G	+++	+++	+++	+++	+++
	3	O	O	++	+++	G	+++	+++	+++	+++	+++
<i>Cladysporium herbarum</i>	1	O	O	G	+++	+	+	+	G	+++	+++
	2	O	O	G	+++	+	++	+	G	+++	+++
	3	O	O	G	+++	+++	++	+	G	+++	+++
<i>Penicillium roqueforti</i>	1	O	O	G	+++	+	++	++	+	++	+++
	2	O	O	+	+++	+++	+++	+++	++	+++	+++
	3	O	O	+	+++	+++	+++	+++	+++	+++	+++
<i>Penicillium camemberti</i>	1	O	O	G	++	G	+	+++	++	+	++
	2	O	O	G	+++	+++	+	+++	+++	+++	+++
	3	O	O	+	+++	+++	+	+++	+++	+++	+++

It will be noted that the first two members of the series are not capable of producing normal cultures. Glycerol is readily available and gives cultures equal in vigor to those grown on cane sugar. With increasing carbon, however, the availability does not increase, as might perhaps be expected. Adonitol, for example, does not compare favorably with glycerol or even erythritol, and two of the hexatomic alcohols fail to yield cultures equal to those on glycerol.

It will be noted that the alcohols beginning with erythritol contain asymmetric carbon atoms. But considering the fact that glycerol is not asymmetric, no connection can be established between availability and carbon asymmetry. On the other hand, there may be some relation between availability and the nature of the intermediate oxidation products, since all the substances which are available, including glycerol, yield oxidation products containing one or more asymmetric carbon atoms.

The writer takes pleasure in acknowledging his indebtedness to Dr. A. W. Dox, at whose suggestion this work was undertaken.



THE COMPARATIVE COMPOSITION OF HUMAN MILK AND OF COW'S MILK.

BY EDWARD B. MEIGS AND HOWARD L. MARSH.

(From the Robert Hare Chemical Laboratory of the University of Pennsylvania and the Wistar Institute of Anatomy and Biology.)

(Received for publication, August 30, 1913.)

INTRODUCTION.

The following article is an account of work done by Arthur V. Meigs, Howard L. Marsh, William H. Welker and W. L. Croll on the chemical analysis of human milk and of cow's milk. The work was carried out in the Robert Hare Chemical Laboratory of the University of Pennsylvania and was to a large extent supervised by John Marshall, the Director of that laboratory. The present account has been written by Edward B. Meigs, in collaboration with Howard L. Marsh, after the death of Arthur V. Meigs, on January 1, 1912.

The subject of milk analysis was taken up by Arthur V. Meigs more than thirty years ago with the idea of discovering how cow's milk should be modified in order to make a proper food for very young infants. On the basis of analyses made in 1881-1884, Meigs devised a food which he afterward used in his practice with great success. He published an account of his early investigations in book form in 1885,¹ and has since published a number of smaller articles on the subject.² In 1908 he again began chemical work on the subject which he continued until his death in 1912. A short preliminary account of some of the results of this later work appeared in 1911;³ and since that time

¹ Arthur V. Meigs: *Milk Analysis and Infant Feeding*, Philadelphia, 1885.

² Arthur V. Meigs: *Transactions of the College of Physicians of Philadelphia*, Third Series, viii, p. 139, 1885; *Ibid.*, xxiv, p. 136, 1902; *Archives of Pediatrics*, December, 1889; *Feeding in Early Infancy*, Philadelphia, 1896.

³ Arthur V. Meigs and Howard L. Marsh: *The Medical Record*, December 30, 1911.

the authors of this article have been endeavoring to coördinate the other results and to prepare them for publication.

Meigs' work on milk in 1881 consisted in an attempt to compare human milk and cow's milk in their content of protein (or "casein"),⁴ fat, lactose, water and salts. The chief outcome of the work was the conclusion that human milk contains less than half as much protein as cow's milk and about 50 per cent more lactose. In other respects Meigs' results were not in sharp disagreement with those of his predecessors, but his figures for protein and lactose were quite different from those which were usu-

TABLE I.

The average composition of human milk and cow's milk according to Meigs' early analyses, and those of certain well-known authors whose results were available in 1881. The various constituents are given as percentages of the whole milk.

AUTHOR	WATER		PROTEIN		FAT		LACTOSE		ASH	
	Human milk	Cow's milk	Human milk	Cow's milk	Human milk	Cow's milk	Human milk	Cow's milk	Human milk	Cow's milk
Lehmann*..			3.5	4.5			4 to 6			
Gorup-										
Besanez†.	88.90	84.28	3.924	4.35	2.666	6.47	4.364	4.34	0.138	0.63
Meigs‡.....	87.16	87.78	1.046	3.022	4.283	3.759	7.407	4.949	0.101	0.488

* Lehmann: *Physiological Chemistry*, Cavendish Society translation, London, 1851, vol. i, p. 383, vol. ii, p. 341.

† Gorup-Besanez: *Lehrbuch der physiologischen Chemie*, Braunschweig, 1878, pp. 421 and 424: the figures for human milk are those quoted from Vernois and Becquerel, and are averages from 89 analyses.

‡ Meigs: *Milk Analysis and Infant Feeding*, Philadelphia, 1885, pp. 34 and 36.

ally given at that time. A comparison of Meigs' figures with some of the best known figures which were available in 1881 is given in Table I.

Meigs was of course familiar with later analyses which had not at that time been quoted in text-books. In these analyses the protein of human milk was variously given at from 0.215 to

⁴ The word "casein" was, at that time, very generally used to designate all the proteins of milk; it now means, as is well known, a particular protein body which constitutes about 80 per cent of the total protein of cow's milk and a somewhat less proportion of the protein of human milk. In the subsequent discussion the words "casein" and "protein" will be used in their modern senses.

over 7 per cent, while the figures for lactose ranged from 1.921 to 8.805 per cent.

In the milk analyses which have been published since 1881, the figures given to represent the percentage of protein and lactose in cow's milk have not varied widely from those published by Gorup-Besanez. But the percentages of these constituents in human milk have been quite variously given, as Table II shows; in the more modern analyses the results are quite near to those reached by Meigs in 1881.

It is generally agreed among physiological chemists at present that the fat, lactose, ash and total nitrogen of milk can be quantitatively determined satisfactorily. The difficulties of milk anal-

TABLE II.

The average composition of human milk according to analyses published since 1881. The constituents are given as percentages of the whole milk.

	WATER	PROTEIN	FAT	LACTOSE	ASH
Munk and Uffelmann*	89.2	2.1	3.4	5.0	0.2
König†	87.4	2.3	3.8	6.2	0.3
Heubner‡		1.03	4.07	7.03	0.21
Camerer and Söldner§		1.27	3.91	6.52	0.22

* Munk and Uffelmann: *Ernährung des gesunden und kranken Menschen*: Wien and Leipzig, 1887, p. 269.

† Quoted by Camerer and Söldner: *Zeitschr. f. Biol.*, xxxiii, 1896, p. 43.

‡ Heubner: *Berl. klin. Wochenschr.*, 1894, Nos. 37 and 38.

§ Camerer and Söldner: *Zeitschr. f. Biol.*, xxxvi, Table II, pp. 280 and 281, 1898. The figures are average figures for milk between the 20th and 40th day post partum. The figure for protein is obtained by multiplying the figure for total nitrogen given by the authors by the factor 6.25.

ysis lie in the determination of the protein and of the unknown constituents. Meigs and his collaborators have, in the first place, determined the quantities of fat, lactose, ash and total nitrogen in a number of samples of human milk and cow's milk; they then attacked the question of the protein and unknown constituents. It will be convenient to divide this article into two parts, in the first of which will be given the results on the easily determinable constituents of milk; and in the second, the work on the protein and unknown substances; this latter work was still in a very fragmentary state at the time of Meigs' death. It will be well to begin with a description of the samples of milk used in the various studies.

Each of the samples of human milk numbered 1, 2, 4, 6, 7, 8 and 10 was made up of milk from several women; each of the other samples was composed of the milk from one woman. The composite samples of human milk came from women in maternity hospitals, and all were taken between the fourth and ninth days after delivery. The other samples of human milk may be described as follows:

No. 3. Colostrum—collected up to and including the fourth day after delivery; maternity hospital patient.

No. 5. Milk collected near the end of lactation, eighteen months or more post partum; woman in moderate circumstances. The flow of milk had almost ceased so that only very little could be obtained.

No. 9. Milk collected during the fifth month after delivery; woman in good circumstances.

The cow's milk came partly from thoroughbred Guernsey cows and partly from grade cows; samples 1, 9, 13 and 20 are the former, the others, the latter. Each of the samples of cows' milk except Nos. 1, 9, 13 and 20 was made up of milk from several cows, and the periods of lactation have not been accurately determined. All the samples may, however, be taken to represent milk from the middle period of lactation, from two to six months after delivery.

In preparing the work for publication, the data obtained from a few samples of milk have been left entirely out of consideration, either because they contained obvious analytical errors, or else because the samples were used for answering subsidiary questions which had no bearing on the composition of milk.

PART I. STUDIES ON THE AMOUNTS OF ASH, LACTOSE, FAT AND NITROGEN IN HUMAN MILK AND IN COW'S MILK.

Methods of experimentation.

The ash determinations were made by igniting the dry residues from the samples cautiously at low temperatures in platinum dishes.

The nitrogen determinations were made by the Kjeldahl method.

The lactose was determined by Fehling's method as follows: 25 cc. of cow's milk or 15 cc. of human milk were diluted to 400 cc. in a 500 cc. graduated flask. Fifteen cc. of $\frac{N}{2}$ NaOH and 10 cc. of CuSO_4 of the strength used in a Fehling's solution were added and the volume of the liquid in the flask was diluted to 500 cc. with water.

After this mixture had been well shaken, it was filtered through a dry filter into a dry flask. One hundred cc. of this solution were added to 50 cc. of hot Fehling's solution and the mixture was boiled for six minutes. It was then quickly filtered through an alundum crucible and washed with about 600 cc. of boiling water. The resulting cuprous oxide in the crucible was dissolved with nitric acid and the solution poured into the beaker which had been used in the reduction. The crucible was then thoroughly washed by passing hot water through it and this wash water was added to

the nitric acid solution which had been poured from the crucible. This nitric acid solution of the cuprous oxide was evaporated on a water bath until free from nitric acid. It was then dissolved with acetic acid and water, 8 grams of zinc acetate were added and the liquid was transferred to a small glass-stoppered bottle, 4 grams of potassium iodide were added and the solution titrated against $\frac{N}{10}$ sodium thiosulphate with starch as an indicator.

The amount of lactose corresponding to the amount of copper found was ascertained by referring to a table given in Bulletin No. 107 (revised) of the U. S. Department of Agriculture, Bureau of Chemistry, pp. 48 and 49. The figures represent lactose plus one molecule of water of crystallization.

Fat was determined by a modification of the method recommended by Meigs in 1885.⁵ To 10 cc. of milk in a 100 cc. glass-stoppered cylinder were added 20 cc. of distilled water and 20 cc. of ethyl ether and the mixture shaken for five minutes. Twenty cc. of 95 per cent ethyl alcohol were then added and the whole again shaken for five minutes. The cylinder was then allowed to stand until its contents separated into two distinct layers. The upper layer was removed by the specially designed pipette shown in figure 1, 5 cc. of ethyl ether were added in such a way as to wash down the sides of the cylinder, this was removed and added to the upper layer previously removed, and the washing of the sides of the cylinder and of the top of the lower layer of the mixture was repeated five times in order to remove all the fat. The upper layer from the mixture plus washings was then evaporated to dryness on a water bath and the residue desiccated to constant weight over sulphuric acid at room temperature. The weight of the residue may be taken to be very nearly that of the fat contained in the milk.

The residue in question was in a number of cases treated with dry ether, and it was found that a minute portion of it usually failed to dissolve. This insoluble portion was, however, never as much as 3 per cent of the weight of the original residue; it amounted on the average to about 1 per cent. It was shown by Fehling's and by the phenyl-hydrazine tests to consist largely of lactose.

The fat as obtained by Meigs' method from two portions of a sample of cow's milk (No. 14) was analyzed for nitrogen: 0.0094 per cent and 0.0064 per cent respectively of nitrogen was found. It is probable that a considerable portion of this comes from the ether-soluble lipoids.

In one sample of human milk (No. 8) the ash, as determined in the fat obtained by Meigs' method, was found to be 0.0081 per cent of the weight of the whole milk.

In other experiments the fat as obtained by Meigs' method in certain samples of milk was compared with the fat as obtained by the Soxhlet extraction of the dried solids. These results have already been published:⁶ in twelve determinations on human milk the fat as determined by Meigs'

⁵ Meigs: *Milk Analysis and Infant Feeding*, Philadelphia, 1885.

⁶ Hawk: *Practical Physiological Chemistry*, Philadelphia, 1912, p. 437.

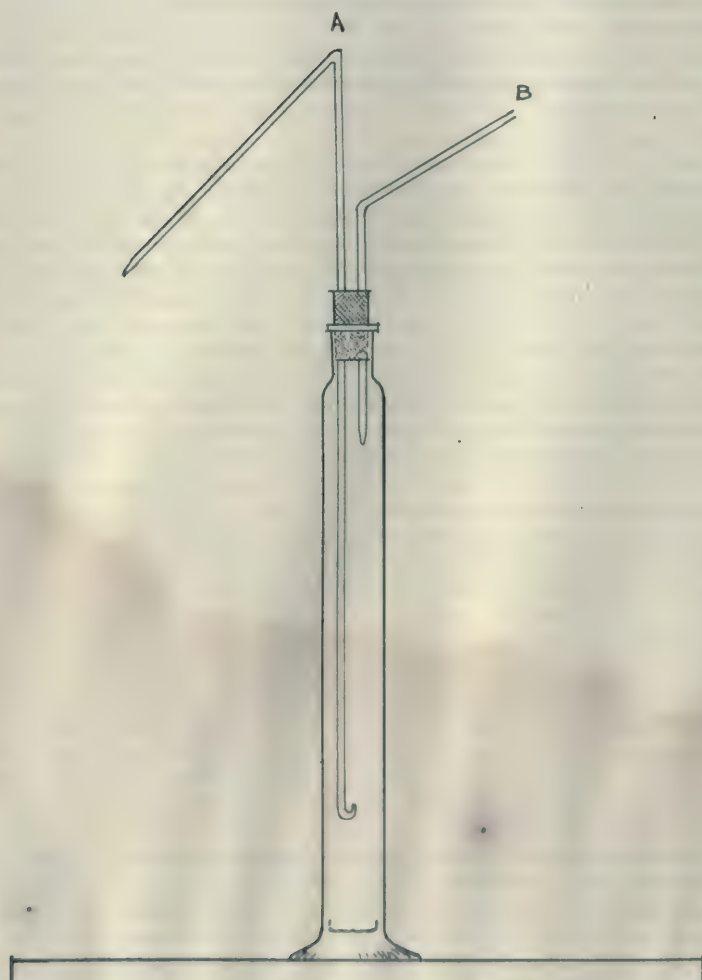


FIG. 1. PIPETTE ARRANGED TO REMOVE THE UPPER LAYER IN MEIGS' METHOD OF FAT EXTRACTION.

The end of the tube A is placed at the surface of the division between the two layers of the mixture of milk treated with the water, alcohol and ether mixture and the upper layer is then forced out through it by forcing air into the space above the mixture through tube B.

method averaged 0.017 per cent less than by the Soxhlet method; and in seven determinations on cow's milk, 0.019 per cent less. It is by no means certain, however, that the figures for fat obtained by the Soxhlet method in these experiments are more nearly correct than those obtained by Meigs' method; for the prolonged treatment of the milk residue with ether in the Soxhlet apparatus is apt to dissolve substances other than fat, and thus give a fictitious additional weight to the fat extract.⁷

The result of this investigation has been that the product obtained by Meigs' method of extracting the fat from milk contains 98 per cent or more of material soluble in dry ether. The results as obtained by Meigs' method are practically the same as those obtained by the Soxhlet extraction; and Meigs' method is much quicker and subjects the milk to less treatment which is apt to produce changes in the protein and carbohydrate constituents of the fluid.

Meigs' method of determining the fat in milk was used as a matter of routine in this investigation, and the figures for fat in the tables represent the dry weight of the material obtained from the "upper layer" and washings in the procedure which has just been described. In some cases, however, the fat was determined by the Soxhlet extraction as well as by Meigs' method. The figures obtained by the Soxhlet extraction are given in a footnote to Table III.

The results of the determinations of the ash, fat, lactose and total nitrogen of human milk and cow's milk are shown in Table III.

These data agree with those of Camerer and Söldner⁸ and with most of the more recent work on milk analysis in showing the marked difference between human milk and cow's milk in respect to their content of nitrogen and lactose. Human milk contains roughly 50 per cent more lactose than cow's milk, and decidedly less than half as much nitrogen. The figures in Table III agree also, so far as they go, with the conclusion of Camerer and Söldner⁹ that the lactose in human milk increases, while the nitrogen decreases with the progress of lactation.

⁷ Croll has published in detail his comparison of the results obtained by Meigs' method of fat extraction and by the Soxhlet method in the *Biochemical Bulletin* for June, 1913.

⁸ Camerer and Söldner: *Zeitschr. f. Biol.*, xxxvi, Table II, pp. 280 and 281, 1898.

⁹ *Ibid.*

TABLE III.

The quantities of ash, fat, lactose and total nitrogen in human milk and cow's milk given as percentages of the weight of the whole milk. Duplicate analyses were carried out on each sample of milk and the table gives the figures obtained in each analysis and the average.

	ASH			FAT			LACTOSE			TOTAL NITROGEN		
	I	II	Average	I	II	Average	I	II	Average	I	II	Average
<i>Human milk.</i>												
(Milk No. 4.....)	0.186	0.189	0.187	2.198	2.210	2.204	6.783	6.759	6.771	0.2209	0.2274	0.2242
Milk No. 6.....	0.191	0.171	0.181	2.234	2.217	2.225	6.443	6.443	6.443	0.2766	0.2765	0.2765
Milk No. 7.....	0.151	0.154	0.152	2.863	2.874	2.868	6.379	6.378	6.379	0.1962	0.1951	0.1956
Milk No. 8.....	0.202	0.191	0.196	2.037	2.019	2.028	6.504	6.504	6.504	0.2642	0.2602	0.2622
Milk No. 10.....	0.249	0.256	0.252	3.147	3.134	3.140	6.600	6.549	6.574	0.2860	0.2828	0.2844
Milk No. 9, 5th month post partum.....	0.185	0.175	0.180	4.081	4.081	4.081	7.067	7.094	7.080	0.1645	0.1574	0.1609
Milk No. 5, collected near the end of lactation.....	0.165	0.162	0.163	0.856	0.850	0.853	7.294	7.305	7.299	0.1965	0.1956	0.1960
<i>Cow's milk.</i>												
{ No. 7.....	0.686	0.676	0.681	4.152	4.139	4.145	4.599	4.641	4.620	0.5288	0.5321	0.5304
{ No. 8.....	0.720	0.721	0.720	4.176	4.224	4.200	4.632	4.601	4.616	0.5500	0.5530	0.5515
Samples of mixed milk from { No. 11.....	0.719	0.660	0.689	3.947	3.936	3.941	4.745	4.737	4.741	0.5600	0.5583	0.5591
{ No. 15.....	0.692	0.694	0.693	4.383	4.384	4.383	4.823	4.823	4.823	0.5042	0.5083	0.5062
{ No. 16.....	0.725	0.731	0.728	3.785	3.804	3.794	4.713	4.713	4.713	0.4981	0.4970	0.4975
{ No. 19.....	0.732	0.735	0.733	3.215	3.220	3.220	4.490	4.474	4.482	0.5269	0.5191	0.5230
Samples of milk from single { No. 1*.....	0.728	0.654	0.691	5.382	5.409	5.395	4.940	5.070	5.005	0.5692	0.5555	0.5623
{ thoroughbred { No. 9.....	0.729	0.739	0.734	4.695	4.689	4.692	4.805	4.819	4.812	0.4959	no dup.	0.4959
{ cows { No. 13.....	0.774	0.768	0.771	4.605	4.615	4.610	4.741	4.755	4.748	0.5948	0.5949	0.5948

* The figures obtained for fat in this sample of milk by the Soxhlet method of extraction were I, 5.319; II, 5.344; average, 5.331.

PART II. THE NATURE AND AMOUNT OF THE PROTEIN AND UNKNOWN SUBSTANCES IN HUMAN MILK AND IN COW'S MILK.

Meigs hoped in his later work to satisfactorily settle the quantities of protein in human milk and in cow's milk, and perhaps even to determine the nature of some of the more important unknown substances, which are supposed to be present in the two secretions. Both these hopes were still unfulfilled at the time of his death, but the work has thrown some further light on the problem; and the results, so far as they go, will be given here.

Experiments which bear solely on the protein content of milk.

The casein and globulin were precipitated from several samples of human milk and cow's milk by magnesium sulphate and the albumin was precipitated from the magnesium sulphate filtrates by acetic acid and heat.¹⁰ The nitrogen content was determined in each precipitate separately, and also, in a separate portion, for the whole milk. The results are given in Table IV.

In other samples of milk the colloids were completely precipitated from the whole milk according to Marshall's¹¹ aluminium hydroxide method. The nitrogen in the colloid precipitates, in the filtrates, and in separate portions of the whole milk was determined. The results are given in Table V.

No very definite conclusions regarding the usual protein content of human milk can be drawn from this part of the work, because the samples of human milk used were so few and from so early a period of lactation. So far as the results go, however, they confirm the view that human milk contains less than half as much protein as cow's milk. The results are interesting also in showing that a large proportion of the nitrogen in early human milk exists in compounds which are not precipitable either by magnesium sulphate or by acetic acid and heat; and the results with Marshall's reagent indicate that most of this "non-precipitable" nitrogen exists in non-colloidal bodies.

¹⁰ For details of these methods see Bulletin No. 107 (revised) of the U. S. Department of Agriculture, 1910, p. 118.

¹¹ Marshall and Welker: *Journ. Amer. Chem. Soc.*, xxxv, p. 820, 1913.

Composition of Milk

TABLE IV.

The total nitrogen, the amounts of nitrogen contained in the casein and globulin precipitates and in the albumin precipitates, and the non-precipitable nitrogen in human milk and cow's milk given as percentages of the weight of the whole milk.*

	TOTAL NITROGEN			NITROGEN IN CASEIN AND GLOBULIN PRECIPITATED BY MAGNESIUM SULPHATE			NITROGEN IN ALBUMIN PRECIPITATED BY ACETIC ACID AND HEAT		NITROGEN NOT PRECIPITATED EITHER BY MAGNESIUM SULPHATE OR BY ACETIC ACID AND HEAT
	I	II	Average	I	II	Average	I	II	Average
<i>Human milk.</i>									
Sample No. 3 (colostrum).....	0.2885	0.3038	0.2962	0.1600	0.1545	0.1572	0.0437	0.0591	0.0514
Sample No. 2 (early milk).....	0.2181	0.2191	0.2186	0.1408	0.1347	0.1377	0.0228	0.0307	0.0267
<i>Cow's milk.</i>									
Sample No. 1 from Guernsey cow.....	0.5692	0.5555	0.5623	0.4970	0.4880	0.4925	0.0469	0.0495	0.0482
[No. 2.....	0.5121	0.5149	0.5135	0.4129	0.4109	0.4119	0.0593	0.0579	0.0586
No. 3.....	0.5355	0.5029	0.5192	0.4347	0.4454	0.4402	0.0564	0.0499	0.0531
[No. 4.....	0.5129	0.5251	0.5190	0.4189	0.4304	0.4246	0.0527	0.0504	0.0515
No. 5.....	0.5251	0.5019	0.5135	0.4321	0.4137	0.4229	0.0394	0.0458	0.0426

* The figures for the "non-precipitable" nitrogen are obtained by subtracting the sum of the quantities of nitrogen in the casein and globulin and in the albumin precipitates from the total nitrogen.

TABLE V.

The total nitrogen contents and the amounts of nitrogen contained in the precipitates by Marshall's method and in the filtrates therefrom in human milk and cow's milk given as percentages of the whole milk.

	TOTAL NITROGEN			NITROGEN IN MARSHALL'S PRECIPITATE			NITROGEN IN FILTRATE FROM MARSHALL'S PRECIPITATION		
	I	II	Average	I	II	Average	I	II	Average
<i>Human milk.</i>									
Sample No. 3 (colostrum).....	0.2885	0.3038	0.2962	0.2343	0.2253	0.2298	0.0724	0.0744	0.0734
Sample No. 2 (early milk).....	0.2181	0.2191	0.2186	0.1699	0.1728	0.1713	0.0496	0.0505	0.0500
<i>Cow's milk.</i>									
Samples of mixed milk	{ No. 2...	0.5121	0.5149	0.5135	0.4791	0.4839	0.0380	0.0385	0.0382
	{ No. 3...	0.5355	0.5029	0.5192	0.5035	0.5008	0.0406	0.0369	0.0388
	{ No. 4...	0.5129	0.5251	0.5190	0.5213	0.4983	0.0190	0.0226	0.0208
	{ No. 5...	0.5251	0.5019	0.5135	0.4732	0.4764	0.0350	0.0340	0.0345

Experiments bearing on the amount and nature of the unknown material contained in milk as well as upon the protein content.

Camerer and Söldner subtracted the sum of the quantities of lactose, fat, ash and citric acid¹² in their samples of milk from the amount of total solids, and called the remainder so obtained the quantity of the "protein plus unknown substances." They found that the nitrogen in cow's milk made up about 16 per cent of the quantity of "protein plus unknown substances," while that in human milk made up only from 11 to 12 per cent.¹³ They then precipitated the protein from milk by alcohol according to Munk's method, and found that the dry ash-free protein of cow's milk contained 14.54 per cent of nitrogen; while that from human milk contained 13.64 per cent.¹⁴ From all these results they concluded that human milk contains a considerable amount of unknown substances which contain little or no nitrogen.

Camerer and Söldner found, as have other investigators, that alcohol does not precipitate the nitrogenous substances from milk so completely as does tannic acid; further, the filtrate from the alcohol precipitation gives a positive reaction with the biuret test and with Millon's reagent. They have studied the non-fatty material which goes into solution in alcohol when the proteins are precipitated from milk by Munk's method and find that it is a soft, sticky material very slightly soluble in water but highly soluble in dilute alkali. Dilute hydrochloric acid gives a white flocculent precipitate from the alkaline solutions. The substance contained about 13 per cent of nitrogen, 0.2 per cent of phosphorus and 1 per cent of ash. From the high nitrogen and phosphorus contents Camerer and Söldner suspect that the material is altered casein.¹⁵

Meigs and his collaborators have gone over the ground covered by Camerer and Söldner on the protein and unknown substances in milk: Their most important results are given in Tables VI and VII.

¹² Camerer and Söldner: *Zeitschr. f. Biol.*, xxxvi, p. 278, 1898.

¹³ Camerer and Söldner: *ibid.*, xxxiii, p. 549 and pp. 562-565, 1896.

¹⁴ Camerer and Söldner: *ibid.*, xxxiii, p. 548, 1896; The alcohol precipitate from the cow's milk contained, on the average, 11.06 per cent of ash; that from the human milk, 6.29 per cent.

¹⁵ Camerer and Söldner: *ibid.*, xxxiii, p. 561, 1896.

TABLE VI.

The quantities of total solids and of "protein plus unknown substances" in human milk and cow's milk given as percentages of the weight of the whole milk; and the percentages of nitrogen which may be supposed to be contained in the "protein plus unknown substances."

		TOTAL SOLIDS			PROTEIN PLUS UNKNOWN SUBSTANCES	PERCENT- AGE OF NITROGEN IN PROTEIN PLUS UNKNOWN SUB- STANCES*
		I	II	Average		
<i>Human milk.</i>						
5th to 9th day post partum...	Milk No. 4.	11.118	11.128	11.123	1.911	11.73
	Milk No. 6.	11.238	11.285	11.263	2.364	11.70
	Milk No. 7.	11.002	11.064	11.033	1.584	12.35
	Milk No. 8.	10.997	10.997	10.997	2.219	11.82
	Milk No. 10.	12.446	12.416	12.431	2.415	11.78
Milk No. 9, 5th month post partum		12.670	12.660	12.665	1.274	12.63
Milk No. 5, collected near end of lactation.....		9.604	9.569	9.586	1.221	16.05
<i>Cow's milk.</i>						
Samples of mixed milk from grade cows.....	No. 7.....	13.320	13.471	13.395	3.769	14.07
	No. 8.....	13.437	13.484	13.460	3.744	14.73
	No. 11.....	13.195	13.188	13.191	3.640	15.36
	No. 15.....	13.343	13.313	13.328	3.249	15.58
	No. 16.....	12.459	12.503	12.481	3.066	16.23
No. 19.....		11.688	11.754	11.721	3.106	16.84
Samples of milk from single thoroughbred Guernsey cows.	No. 1.....	15.265	15.230	15.247	3.976	14.14
	No. 9.....	14.080	14.104	14.092	3.674	13.50
	No. 13.....	14.035	14.033	14.034	3.725	15.97

* The average percentage of nitrogen in the "protein plus unknown substances" in samples 4, 6, 7, 8, 9 and 10 of human milk is 12 per cent. Sample 5 is omitted from this calculation, as the milk came from very near the end of lactation and was evidently abnormal. The average percentage of nitrogen in the "protein plus unknown substances" in the samples of cow's milk is 15.18 per cent.

Table VI gives the amount of total solids and of "protein plus unknown substances" in a number of samples of human milk and of cow's milk. The figures for the "protein plus unknown substances" were obtained in the same way as those of Camerer and Söldner. The average nitrogen contents of the "protein plus unknown substances" as calculated from our figures is 12 per cent in the case of human milk and 15.18 per cent in the case of cow's milk.

TABLE VII.

The dry weight of protein precipitated by alcohol from fat-free human milk and cow's milk given as percentages of the weight of the whole milk; the amount of nitrogen contained in the alcohol precipitate given as percentage of the weight of the whole milk; and the percentages of nitrogen contained in the dry alcohol precipitates.

DRY WEIGHTS OF ALCOHOL PRECIPITATES*			AMOUNTS OF NITROGEN CONTAINED IN ALCOHOL PRECIPITATES GIVEN AS PERCENTAGES OF WHOLE MILK			AMOUNTS OF NITROGEN CONTAINED IN ALCOHOL PRECIPITATES GIVEN AS PERCENTAGES OF DRY ASH-FREE PRECIPITATES				
I	II	Average	I	II	Average	I	II	Average		
<i>Human milk.</i>										
Precipitated by 75 per cent alcohol	Milk No. 4	1.064	1.052	1.058	0.1506	0.1530	0.1518	14.15	14.54	14.35
	Milk No. 5	0.911	0.869	0.890	0.1465	0.1468	0.1466	16.08	16.89	16.47
	Milk No. 8	1.292	1.315	1.303	0.1666	0.1666	0.1666	12.90	12.67	12.78
Precipitated by 86 per cent.	Milk No. 9	0.786	0.793	0.789	0.1049	0.1024	0.1036	13.35	12.91	13.13
	Milk No. 10	1.440	1.428	1.434	0.1941	0.1970	0.1955	13.48	13.80	13.64
Precipitated by 90 per cent alcohol	Milk No. 7	0.991	0.957	0.974	0.1474	0.1471	0.1472	14.87	15.37	15.11
<i>Cow's milk.</i>										
Precipitated by 75 per cent alcohol	Milk No. 7	3.450	3.375	3.412	0.4800	0.4740	0.4770	13.91	14.03	13.97
	Milk No. 8	3.429	3.392	3.410	0.4878	0.4949	0.4913	14.23	14.60	14.41
	Milk No. 9	3.375	3.412	3.393	0.4596	0.4636	0.4616	13.62	13.59	13.60
Precipitated by 86 per cent alcohol	Milk No. 13	3.814	3.808	3.811	0.5464	0.5451	0.5457	14.33	14.31	14.32
	Milk No. 19	3.013	3.047	3.030	0.4276	0.4253	0.4264	14.19	13.96	14.06

* The figures in this column represent the weight of the ash-free precipitates. The ash in the alcohol precipitates was determined by Marsh in the cases of samples 8, 9, 10 and 7 of human milk, and in the cases of samples 9, 13 and 19 of cow's milk; it was found to be 0.058, 0.079, 0.084 and 0.066 in the human milk and 0.414, 0.526, and 0.393 in the cow's milk respectively—on the average 6 per cent of the weight of the whole precipitate in human milk and 11 per cent of the weight of the whole precipitate in cow's milk. In the remaining samples of human milk the ash-free precipitate is reckoned to be 94 per cent of the weight of the ash-containing precipitate; in the remaining samples of cow's milk, 89 per cent. See also Camerer and Söldner: *Zeitschr. f. Biol.*, xxxiii, Table III, p. 548, 1896.

The dry weight of the material precipitated from samples of human milk and cow's milk by alcohol and the nitrogen content of this material are given in Table VII. The ash-free protein precipitated from human milk by alcohol contained on the average 13.80 per cent of nitrogen; while that from cow's milk contained 14.07 per cent.

The following is an account of the methods used by Meigs and his collaborators in this part of the investigation.

Samples of milk were placed in platinum dishes (without sea sand) and were kept in a water oven at about $98^{\circ 16}$ until ten hours after the visible fluid in them had disappeared. The residues were then placed in desiccators over sulphuric acid at room temperature until they reached constant weight, and this figure was taken as the weight of the total solids. The lactose retains its water of crystallization under these conditions of drying.¹⁷

The protein precipitated from milk by alcohol was determined as follows: The fat was removed from 10 cc. of milk by Meigs' method described on pages 151-153. After the removal of the fat had been completed the lower layer of the mixture (which contained the non-fatty constituents from 10 cc. of milk) was transferred to a 200 cc. beaker and evaporated on a water bath to a volume of about 10 cc.; 95 per cent alcohol was then added. The amount of alcohol added differed in different experiments; in some cases the mixture obtained by adding the alcohol contained 75 per cent of alcohol; in others, 86 per cent; and in still others, 90 per cent of alcohol. The mixture was allowed to stand five minutes and then filtered through a weighed porous alundum crucible by means of a suction pump and washed with 1 liter of boiling alcohol and subsequently with 500 cc. of ether. Before being washed, the protein was carefully broken up into small particles with a glass rod.

The contents of the crucible were dried first in a water oven at 98° and then in a desiccator over sulphuric acid at room temperature until they reached constant weight. They were then weighed, and their content of nitrogen was afterwards determined.

The filtrates from the milk treated with alcohol as described above were studied in various ways. It was found that if these filtrates were evaporated to small volume and then treated with a considerable quantity of water, there was precipitated from them a material which resembled that described by Camerer and Söldner (see page 158). Marsh determined the solubilities of this material

¹⁶ Temperatures are throughout this article expressed in terms of the centigrade scale.

¹⁷ Camerer and Söldner: *Zeitschr. f. Biol.*, xxxiii, pp. 538-540, 1896.

and the nitrogen content. He found that all of it was soluble in alcohol and only a portion in ether. The ether-insoluble portion of the material is insoluble in water and in acids; soluble in alcohol, chloroform and alkalies. It gives a positive reaction to both Millon's test and the biuret. It contains 13.8 per cent of nitrogen. On being ignited, it leaves a little ash which contains phosphorus. It can be obtained in larger quantities from human milk than from cow's milk.

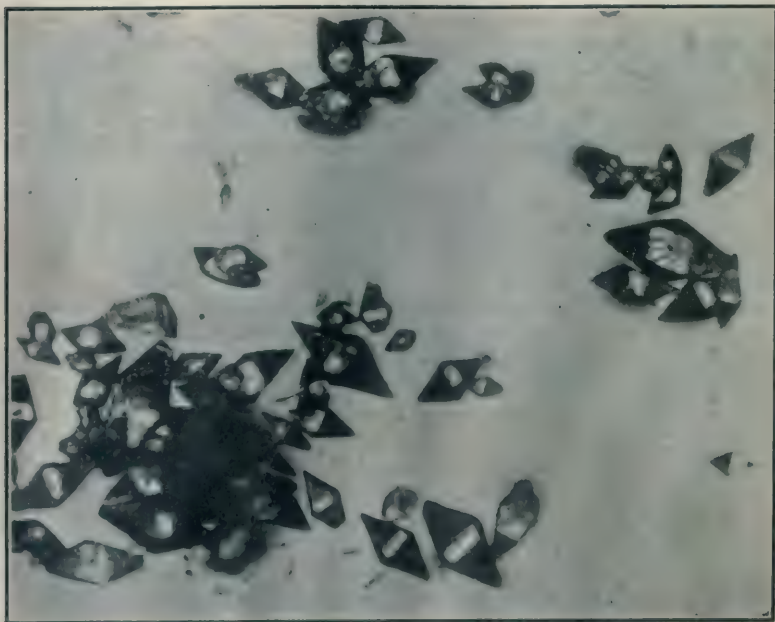


FIG. 2. CRYSTALS, OF SUBSTANCE OF UNKNOWN COMPOSITION FROM HUMAN MILK.

It has been found possible to obtain two kinds of crystals from the alcohol filtrate of human milk treated as described on page 161. Of these one appears as long glistening prisms. These crystals are soft and represent, in all probability, a minute remnant of fat, which had not been removed by Meigs' method of fat extraction. The appearance of the other kind of crystal is shown in Figure 2, which is reproduced from a photograph.

It was afterward found that the crystals shown in Figure 2 could most easily be obtained as follows:

The fat was removed from samples of milk by Meigs' method described on pages 151-153; the fat-free or nearly fat-free lower layer was then shaken up with ether; the mixture allowed to stand; and the upper layer, consisting of a mixture of alcohol and ether, was drawn off. This was evaporated over boiling water to a volume of about 20 cc. and then allowed to evaporate spontaneously. The crystals usually made their appearance before the supernatant fluid had entirely evaporated. The production of the crystals has, however, been rather capricious; no method has yet been devised by which they could be obtained with certainty.

We have submitted these crystals to Dr. A. P. Brown, Professor of Geology and Mineralogy in the University of Pennsylvania, for examination and have obtained the following report:

The crystals belong to the Tetragonal System.

Their axial ratio is $a : c = 1 : 2.46$.

Form observed: The unit pyramid was the only form present.

Angles: The plane angle of the face of the unit pyramid, between the pole edges $1\bar{1}1 - 111 \wedge 111 - \bar{1}11 = 35^\circ 20'$; also the edge angle over the pole, edges $111 - \bar{1}11 \wedge 1\bar{1}1 - \bar{1}11 = 44^\circ 20'$ (calculated $44^\circ 6'$).

Habit of crystals: The unit pyramid is alone developed; sometimes symmetrically, more often distorted by contact with the surface upon which it rests, giving the appearance at first sight of the unit pyramid combined with the basal pinacoid (001) and with the unit prism (110), but these forms are not seen. No twins were observed, but parallel growths of the unit pyramids were fairly common. The crystals are brittle; no cleavage was observed.

The refractive index is high and the double refraction is very strong. Examined in polarized light, the extinction is straight in all ordinary positions. The greater refractive index is for light vibrating parallel to the crystal axis, c ; hence $\epsilon > \omega$, or the crystal is optically positive (+). On examining the crystal in the direction of the vertical axis, c , in convergent polarized light, the uniaxial interference figure is observed, thus confirming the tetragonal character.

As a check on the angles, the angle over the pole was measured, angle $111 \wedge \bar{1}11 = 32^\circ 40'$ as against $32^\circ 4'$ calculated. The profile view of the crystal lying on the pole edge gave an edge angle over the pole of about 54° .

These crystals are fairly soluble in ether, somewhat soluble in 95 per cent alcohol, and nearly insoluble in water, though treatment with water alters them, rendering them opaque and in time destroying their crystalline form. They dissolve very slowly in acetone. They have been submitted to Lassaigne's test for nitrogen, and thereby shown to be free from nitrogen; but the appli-

cation of the plumbic acetate test¹⁸ shows that they contain a considerable amount of sulphur.

They represent a substance, which, so far as we know, has not been heretofore described as a component of milk.

The immediately preceding paragraphs may be summed up by saying that the results of Meigs and his collaborators on the protein and unknown substances of milk are throughout in close agreement with those of Camerer and Söldner. Both sets of observers find that the total nitrogen in human milk makes up a decidedly smaller proportion of the "protein plus unknown substances" than it does in the case of cow's milk; both sets of observers find that the materials precipitated from the two kinds of milk by alcohol have about the nitrogen content which is usual for protein; finally, both sets of observers obtained similar materials from the alcohol filtrates. This agreement is made all the more striking by the fact that neither Meigs nor any of his collaborators was familiar with the work of Camerer and Söldner at the time when their experiments were carried out. They obtained their results quite independently. We are inclined, therefore, to regard it as established, or at least very highly probable, that human milk contains a considerable amount of unknown material, which has either a low nitrogen content or else none at all of that element.

What becomes of this material when milk is subjected to analysis? It has been shown above that unknown substances can be extracted from milk by means of alcohol, and we shall consider very briefly the question whether the material of the alcohol extract is to any extent identical with the "x material," the existence of which in human milk can be inferred from the work of Camerer and Söldner and of Meigs and his collaborators.

Camerer and Söldner advance the hypothesis that the material of the alcohol extract is "altered casein." We think this improbable for the following reason. Cow's milk contains about three times as much casein as human milk. If, therefore, alcohol alters casein so that a part of it becomes alcohol-soluble, more of this altered casein should appear in the alcohol filtrate from

¹⁸ Hawk: *Practical Physiological Chemistry*, 4th edition, Philadelphia, 1912, pp. 108, 109.

cow's milk than in that from human milk. But just the opposite is the case.

We have endeavored to get an approximate idea of the total amount of unknown material in the alcohol extract from cow's milk and human milk. Two samples of human milk and three samples of cow's milk were freed from fat by Meigs' method, and the protein was precipitated from the fat-free residue by 86 per cent alcohol.

The filtrates from the 86 per cent alcohol protein precipitations were then evaporated to dryness and the weights of the ash-free residues were determined. These residues contained all the lactose from the milk plus the unknown alcohol-soluble material. In other portions of the same samples of milk the lactose was determined by the Fehling method—see Table III. Subtracting the amounts of lactose from those of the ash-free residues in the five samples of milk gives the following results:

		<i>Percentage of ash-free alcohol residue</i>		<i>Percentage of lactose</i>		<i>Percentage of unknown alco- hol-soluble material</i>
Human milk..	{ No. 10....	7.952	—	6.574	=	1.378
	{ No. 9.....	7.575	—	7.080	=	0.495
Cow's milk...	{ No. 16....	5.104	—	4.713	=	0.391
	{ No. 19....	5.068	—	4.482	=	0.586
	{ No. 13....	4.935	—	4.748	=	0.187

The following is another method by which an approximate idea of the amount of unknown alcohol-soluble material in milk may be gained. In the samples of human milk Nos. 4, 5, 7, 8, 9 and 10 the weights of the material precipitated by alcohol have been determined (Table VII) and may be compared with the quantities of "protein plus unknown substances" found in Table VI. It will be seen that the quantities of the latter are always considerably larger than those of the former; the results obtained by subtracting the quantities of ash-free alcohol precipitates from those of the "protein plus unknown substances" are as follows: Milk No. 4, 0.853; No. 7, 0.610; No. 8, 0.916; No. 10, 0.981; No. 9, 0.485; No. 5, 0.331. The same data for the samples of cow's milk, Nos. 7, 8, 9, 13 and 19 are given in Tables VI

and VII; and the results of similar calculations are as follows: Milk No. 7, 0.357; No. 8, 0.334; No. 9, 0.281; No. 19, 0.076.¹⁹

The figures which have just been given to represent the amounts of the unknown alcohol-soluble material in human milk at different periods of lactation correspond very fairly with the figures for the amounts of the x material at the corresponding periods of lactation as calculated from the data of Camerer and Söldner and from those of Meigs and his collaborators. It seems probable, therefore, that the alcohol-soluble material is to a considerable extent identical with the x material. That it is not wholly identical with the x material is evident from general considerations as well as from its high nitrogen content. Milk contains such bodies as urea, ammonia,²⁰ and purine bases²¹ which would not be precipitated by alcohol; and the nitrogen in these would account for a considerable part of the nitrogen in the alcohol-soluble material.

The known alcohol-soluble substances in milk.

Koch and Woods²² find 0.036 to 0.049 per cent lecithin and 0.027 to 0.045 per cent kephalin in cow's milk—0.072 to 0.086 per cent total "lecithans."²³ For human milk the figures of these investigators are lecithin, 0.041 per cent; kephalin, 0.037 per cent; total lecithans, 0.078 per cent.

Raudnitz²⁴ gives a review of previous determinations of cholesterol²⁵ in milk. Tolmatscheff found 0.0252 to 0.0385 per cent of this substance in human milk; Schmidt-Mülheim was able to demonstrate its presence in cow's milk. Marsh determined the amount of cholesterol in cow's milk according to the method of Ritter²⁶ and found 0.021 per cent.

¹⁹ In the case of sample No. 13 of cow's milk there was some error in analysis so that the weight of the material precipitated by alcohol appeared to be slightly greater than that of the "protein plus unknown substances."

²⁰ Camerer and Söldner: *Zeitschr. f. Biol.*, xxxvi, p. 299, 1898.

²¹ Raudnitz: *Ergeb. d. Physiol.*, 1903, ii, p. 257.

²² Koch and Woods: *This Journal*, i, p. 211, 1906.

²³ By "lecithans" Koch and Woods mean the phosphorus-containing lipoids.

²⁴ Raudnitz: *Ergeb. d. Physiol.*, 1903, ii, p. 264.

²⁵ Raudnitz uses the older term, "cholesterin."

²⁶ Ritter: *Zeitschr. f. physiol. Chem.*, xxxiv, p. 430, 1901-02.

The substances which have been spoken of above—lecithin, kephalin, cholesterol, urea, ammonia and the purine bases—would account for less than one-fifth of the unknown material in the alcohol extract of human milk.

It is realized that the data given above and bearing on the quantity of the material in the alcohol extract of milk are very incomplete. But they do indicate that human milk contains considerably more non-fatty, alcohol-soluble material than cow's milk; and it has been thought worth while to present them in view of recent interesting work on the importance of unknown substances in milk as accessory factors in diet. Stepp,²⁷ for instance, has found that mice always die in a few weeks when given food materials which have been fully extracted with alcohol and ether, and that the diet may be rendered again capable of sustaining life by the addition of the material from the alcohol-ether extract of skimmed milk. Stepp has been able to show that the material necessary to maintain life is not either fat, cholesterol, lecithin or salts.

GENERAL CONCLUSIONS.

Human milk differs from cow's milk in three important ways. It contains considerably more lactose than cow's milk, and more substances of unknown nature which contain little or no nitrogen; it contains very much less protein than cow's milk. The composition of milk varies more or less regularly with the progress of lactation so that average figures for its composition are not very satisfactory. The following, however, may be taken as the limits of normal variation of the constituents of the two kinds of milk from the beginning of the second month of lactation onward, the figures representing percentages of whole milk:

	FAT	LACTOSE	PROTEIN
Human milk.....	2-4	6-7.5	0.7-1.5
Cow's milk.....	2-4	3.5-5	2.5-4

Both kinds of milk contain substances which are important constituents of diet, which are soluble in alcohol and ether, which

²⁷ Stepp: *Zeitschr. f. Biol.*, lvii (N. F. xxxix), p. 135, 1911.

contain little or no nitrogen, but of which the chemical nature is still unknown. These substances are most plentiful in early human milk and diminish in amount with the progress of lactation. Early human milk contains about 1 per cent of these unknown substances; milk from the middle period of lactation about 0.5 per cent. Cow's milk from the middle period of lactation contains about 0.3 per cent of the unknown substances.

THE INFLUENCE OF THE ADMINISTRATION OF CREATINE AND CREATININE ON THE CREATINE CONTENT OF MUSCLE.

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With the appearance of an accurate method of estimating creatinine, the story of the origin of this interesting urinary constituent has been found to be less simple than was formerly supposed. Without doubt, the information which has been brought to light with its aid has been very important, although in many instances this has been contrary to the previously accepted views, and difficult of satisfactory explanation. That the creatinine of the urine has its origin in the creatine of the muscle seems obvious on *a priori* grounds, but the satisfactory demonstration of this has been beset with unforeseen difficulties.¹ Folin,² with the aid of his colorimetric method, was the first to point out that the quantitative conversion of creatine to creatinine or creatinine to creatine *in vitro* was far more difficult than previous statements would lead one to believe. That the body was even less able to bring about a quantitative conversion of creatine to creatinine was shown by his feeding experiments on man. In fact he was unable to adduce any evidence of a conversion of the administered creatine to creatinine. Further experimental data bearing on the fate of administered creatine and creatinine have been presented by Klercker,³ Wolf and Shaffer,⁴ van Hoogenhuyze and Verploegh,⁵

¹ For an interesting critical review of the general subject of creatine-creatinine metabolism, reference may be made to the paper of Riesser: *Zeitschr. f. physiol. Chem.*, lxxxvi, p. 415, 1913.

² Folin: *Hammarsten's Festschrift*, III, 1906.

³ Klercker: *Beitr. z. chem Physiol. u. Path.*, viii, p. 59, 1906; *Biochem. Zeitschr.*, iii, p. 45, 1907.

⁴ Wolf and Shaffer: this *Journal*, iv, p. 439, 1908.

⁵ van Hoogenhuyze and Verploegh: *Zeitschr. f. physiol. Chem.*, lvii, p. 161, 1908.

Lefmann,⁶ Plimmer, Dick and Lieb,⁷ Pekelharing and van Hoogenhuyze,⁸ Foster and Fisher,⁹ Towles and Voegtlin,¹⁰ Folin and Denis,¹¹ and Kraus.¹² It has been observed that when creatinine is administered to man or animals, either *per os* or parenterally, about 80 per cent reappears in the urine during the succeeding twenty-four hours. After the administration of creatine, the excretion of creatinine has been found to be only slightly increased, if at all. When given in comparatively large amounts a considerable portion may reappear in the urine as such, though with small amounts it does not reappear. In contradistinction to creatinine, Folin has viewed the creatine under these conditions not as a waste product but as a food.

Since the administered creatine cannot be accounted for by the creatine (and creatinine) of the urine, it seems logical to assume that this remaining portion is either metabolized or stored up in the muscle. This last possibility was considered by Mellanby¹³ in experiments on rabbits and chickens. He concludes, "Creatine and creatinine feeding has no effect upon the creatine content of muscle after the muscle has reached a certain saturation point. There is some evidence of an increase in muscle creatine at an early stage of life on feeding with creatine and possibly with creatinine." In an endeavor to throw light upon the origin of creatine, Riesser,¹⁴ in his recent studies, observed a slight increase in the concentration of muscle creatine after the administration of betaine and choline.

The possibility that the creatine, which does not reappear in the urine after its introduction into the body, is deposited in the muscle tissue, has not been answered, and it was primarily for this reason that the present experiments were undertaken. In his experiments Mellanby took no account of the portion of the ad-

⁶ Lefmann: *Zeitschr. f. physiol. Chem.*, lvii, p. 476, 1908.

⁷ Plimmer, Dick and Lieb: *Journ. of Physiol.*, xxxix, p. 98, 1909.

⁸ Pekelharing and van Hoogenhuyze: *Zeitschr. f. physiol. Chem.*, lxix, p. 395, 1910.

⁹ Foster and Fisher: *this Journal*, ix, p. 359, 1911.

¹⁰ Towles and Voegtlin: *ibid.*, x, p. 479, 1912.

¹¹ Folin and Denis: *ibid.*, xii, p. 148, 1912.

¹² Kraus: *Arch. of Int. Med.*, xi, p. 613, 1913.

¹³ Mellanby: *Journ. of Physiol.*, xxxvi, p. 470, 1908.

¹⁴ *Loc. cit.*

ministered creatine or creatinine that might be lost in the urine. Obviously, this is a factor of importance in the interpretation of the content of muscle creatine. Such analyses were included in the present experiments, in order that we might ascertain in so far as possible the fate of *all* the administered creatine and creatinine.

METHODS.

The general analytical procedures employed in the analysis of tissue and urine were those previously described.¹⁵ The creatine used in the first experiment (rabbit 47) was prepared from rabbit muscle. In the succeeding experiments, however, the creatine and creatinine were prepared from urine according to unpublished directions supplied us by Prof. S. R. Benedict. With these methods we have prepared about 100 grams of creatine and 10 grams of creatinine, both of the highest degree of purity. Our thanks are due to Professor Benedict for furnishing us with the details of these admirable methods. In all experiments the creatine and creatinine were administered subcutaneously in 1 per cent aqueous solution between the shoulder blades with a 10 cc. Luer glass syringe. In every case the strength of the solution employed was ascertained colorimetrically, although in several experiments this was checked by employing carefully dehydrated preparations, which could be accurately weighed. The time of administration was generally from 2-5 hours after the end of the previous twenty-four-hour period. The injections extended over a period of 5-13 consecutive days. It was believed that in this way the maximum retention of creatine in the muscle would be observed, and further that the average daily composition of the urine during this interval would furnish a very reliable comparison with that of the control period. There appeared to be a lag in the elimination of urine in one or two cases, notably in rabbit 71, although it is improbable that this could have had an important influence upon the average data of a period of several days. Nitrogen determinations were made on the urines of all rabbits with the exception of rabbits 71, 72, 73 and 74. The daily variations were so slight as to fall within the limits of error; and as they are without significance for our present purposes, they have not been included in this paper.

¹⁵ Myers and Fine: this *Journal*, xiv, p. 9, 1913.

EXPERIMENTAL PART AND DISCUSSION.

Eleven injection experiments with creatine and creatinine are reported upon rabbits, eight with creatine and three with creatinine. Two of the eight experiments with creatine (numbers 71 and 72) did not include estimations of the creatine of the muscle, while one experiment, number 57, was carried out on a rabbit fed upon a practically pure carbohydrate diet.¹⁶ The important results of these experiments are summarized in Tables I and II, the experimental details being recorded in the Tables III–XII appended to this paper. The creatine employed in experiments 47, 49, 56, 59 and 57 was tested qualitatively for creatinine and failed to give an appreciable reaction. Following the period of injection, the creatinine excretion was found to be slightly increased, the equivalent of a conversion of about 3 per cent of the administered creatine. On this account, three additional experiments, 71, 72, and 73, were conducted employing creatine, the purity of which had been quantitatively ascertained. Four colorimetric tests were made with this preparation during the course of the experiments, employing in each case 0.1-gram portions. The colorimetric readings indicated that the possible contamination of creatinine was not over 0.15 to 0.20 per cent, an amount insignificant in this connection. It is interesting to note that even with this very pure preparation of creatine there was an increased elimination of creatinine, if anything greater than that observed in the earlier trials.

The data on the influence of the administration of creatine and creatinine on the creatine content of the muscle are summarized in Table I. The results for the nitrogen and moisture content of the muscle are very uniform and all fall within normal limits. The figures for the creatine content are, however, uniformly above the usual normal figure of 0.52 per cent, if we except carbohydrate rabbit 57; and even here the creatine content is considerably higher than in other animals fed carbohydrate but no creatine.¹⁷ The increase after the creatine injections in the first five experiments amounts to about 5 per cent.¹⁸ It is true that this increase is slight

¹⁶ See Myers and Fine: *this Journal*, xv, p. 305, 1913.

¹⁷ *Loc. cit.*

¹⁸ This is the percentage increase over the normal 0.522 per cent. The absolute amount of creatine retained by the body is calculated by mul-

TABLE I.

The creatine content of rabbit muscle as influenced by the subcutaneous administration of creatine and creatinine.

ANIMAL	BODY WEIGHT	CREATINE CONTENT OF BODY		COMPOSITION OF MUSCLE			LENGTH OF TIME AFTER LAST INJECTION BEFORE KILLING ANIMAL
		Calculated Body Wt. $\times 0.182^*$	Found	Nitrogen	Moiستure	Creatine	
<i>Experiments with creatine.</i>							
	kgms.	grams	grams	per cent	per cent	per cent	days
47	1.65	3.00	3.02	3.71	76.1	0.544	3
49	1.72	3.13	3.47	3.61	75.9	0.546	3
56	1.81	3.29	3.70	3.35	77.1	0.559	3
59	2.00	3.64	3.90	3.52	76.7	0.553	4
73	1.66	3.02	3.04	3.63	75.3	0.540	1
57†	1.98-1.22	3.60	2.18	3.55	76.2	0.482	1

Experiments with creatinine.

	kgms.	grams	grams	per cent	per cent	per cent	days
58	1.68	3.06	3.49	3.55	76.3	0.540	3
62	1.70	3.02	3.09	3.45	76.4	0.566	4
74	1.93	3.51	4.04	3.59	76.0	0.566	1

* Calculated from average data in Table VII of a previous paper, this *Journal*, xiv, p. 23, 1913.

† See Myers and Fine: *ibid.*, xv, p. 305, 1913.

and we would attach greater significance to the fact that a large amount of creatine was not stored up in the muscle than to the fact that a small amount was retained. This slight increase we believe, however, to be a little beyond the limits of error of the method when carefully carried out. Many colorimetric readings were made and compared with readings from control animals. The controls were found under the conditions of our experiments to give the usual readings of about 9.0 mm., whereas the samples from the animals to which the creatine and creatinine had been administered gave readings varying between 8.3 and 8.7 mm. As a further control, it was found that creatine added to muscle before extraction could be quantitatively recovered under the same conditions. The increase in the creatine content of the

tiplying the increase per gram of muscle by the total muscle tissue of the body. The latter is estimated by dividing the total body creatine by the percentage found.

muscle is, if anything, more pronounced after the administration of creatinine than of creatine. It would not be expected that creatinine would be retained by the muscle unchanged, and in the case of rabbit 74 a creatinine estimation¹⁹ on the fresh muscle showed the presence of about 9 mgms. of creatinine per 100 grams of muscle, an amount not far from the normal. Rabbit 73, to which creatine had been given, contained 7 mgms. creatinine per 100 grams of muscle. It seems evident from the above trials that the creatine content of the muscle tissue can be raised slightly above the ordinary level by the administration of either creatine or creatinine. That creatinine exerts this influence is in harmony with the view that the reaction between these two substances is reversible. This is further borne out by the observations of other workers of an excretion of creatine following the administration of creatinine. The increase in the concentration of muscle creatine in rabbits, which Riesser²⁰ observed after the injection of betaine and choline is quite similar to that observed in our own experiments; and it is further of interest that in his six control animals he obtained figures identical with those which we have observed, viz., 0.52 per cent.²¹

Further evidence of a small retention of creatine is found in the comparison of the total content of creatine in the body calculated from the body weight with that actually found to be present, the latter always being the greater. The error incident to this comparison is, of course, considerable, but, nevertheless, the fact that the figures based upon actual determinations are uniformly higher than those calculated lends support to the above view. It would seem, then, that by the administration of either creatine or creatinine the concentration of muscle creatine may be raised about 5 or 6 per cent above the values ordinarily obtained. That the extra creatine is quite firmly held is indicated by the fact that the concentration of the creatine in the muscle is practically the same whether the rabbit is killed one day or four days after the last injection.

The various factors concerned in the fate of creatine and

¹⁹ The method employed will be described in a subsequent paper.

²⁰ *Loc. cit.*

²¹ Myers and Fine: *this Journal*, xiv, p. 14, 1913.

TABLE II.

The fate of creatine and creatinine when administered subcutaneously in the rabbit.

ANIMAL	BODY WEIGHT	TOTAL AMOUNT OF CREATINE OR CREATININE INJECTED	PERIOD OF INJECTION	CREATINE OR CREATININE INJECTED PER KGM. PER DAY	FATE OF ADMINISTERED MATERIAL			
					Eliminated as creatine in urine	Elim. as creatinine in urine	Retained as creatine in muscle	Unaccounted for

Experiments with creatine.

	kgms.	grams	days	mgms.	per cent	per cent	per cent	per cent
47	1.65	0.95	5	115	60	4	13	23
49	1.72	1.02	12	49	26	7	15	52
56	1.81	1.06	10	59	32	2	23	43
59	2.00	1.22	9	85	67	1	18	14
71	2.55	0.90	6	59	53	10		
72	2.45	0.90	6	61	47	14		
73	1.66	0.70	7	60	81	7	14	0
57	1.98-1.22	1.09	13	52	58	2		

Experiments with creatinine.

	kgms.	grams	days	mgms.	per cent	per cent	per cent	per cent
58	1.68	0.75	9	50	0	82	13	5
62	1.70	0.70	6	69	0	80	28	0
74	1.93	0.90	6	78	0	77	26	0

creatinine when introduced into the body are recorded in Table II. When creatine is administered subcutaneously to rabbits in amounts varying between 50 and 100 mgms. per kilogram of body weight per day, a considerable portion (25-80 per cent) reappears in the urine unchanged. In most of the trials about 3 to 4 per cent appeared in the urine as creatinine, although the amount excreted in this form may be considerably greater, as in rabbits 71 and 72. A possible explanation for these high results of 10 and 14 per cent may be found in the fact that these animals were very large and had "creatinine coefficients" of 16 and 17 respectively. As we have previously pointed out,²² the average creatinine coefficient for the rabbit is 14, and these higher coefficients possibly indicate a greater efficiency on the part of these animals in the conversion of creatine to creatinine.

²² Myers and Fine: this *Journal*, xiv, p. 19, 1913.

Van Hoogenhuyze and Verploegh were apparently the first to call attention to the slightly increased excretion of creatinine after the administration of creatine, an observation confirmed by Pekelharing and van Hoogenhuyze, Towles and Voegtlin, S. R. Benedict²³ and the present writers. Although creatine and creatinine may not have the very simple metabolic relationship formerly supposed, it is not quite obvious how Klercker and Lefmann from their data draw the conclusion that exogenous creatine is not transformed to creatinine at least in small part.

In a previous communication,²⁴ attention was called to the significant fact that the percentage of administered creatine which was converted to creatinine was not widely different from the relationship existing between the daily creatinine of the urine and the total body creatine. It was further suggested that, since creatine is held in such a loose state of combination in the muscle, it is not illogical to believe that it experienced the same fate as the administered creatine.

Under the conditions of our experiments, it appears that about 15 per cent of the injected creatine may be stored in the muscle. The portion of the creatine remaining unaccounted for seems to be chiefly dependent upon the amount of the creatine administered, or, in other words, upon the opportunity given the body to oxidize it. The experimental data in the case of creatinine seem to indicate that the 20 per cent which is not excreted in the urine may be completely stored up in the muscle as creatine.

CONCLUSIONS AND SUMMARY.

The subcutaneous administration of creatine to rabbits appears to cause a small increase in the creatine content of the muscle, about 5 per cent in five experiments. This is quite insufficient, however, to account for the creatine which does not reappear in the urine.

The administration of creatinine appears to exert a similar influence upon the creatine content of the muscle. In three experiments the creatine content was found to be about 6 per cent above the normal, an amount sufficient to account for the creatinine

²³ Private communication.

²⁴ Myers and Fine: *this Journal*, xv, p. 304, 1913.

not eliminated by the kidneys. This apparent increase in the creatine content of the muscle was not due to a retention of the creatinine unchanged.

Of the creatine administered in our experiments, 25-80 per cent—the quantity depending upon the amount injected—reappeared in the urine unchanged, while from 2-10 per cent was eliminated in the form of creatinine. We are inclined to attach considerable significance to this small conversion of creatine to creatinine, as throwing light upon the relationship of these two substances in metabolism.

When creatinine was administered 77-82 (average 80) per cent reappeared in the urine. No elimination of creatine was detected.

TABLE III.
Rabbit 47—Injection of creatine.

DAY	BODY WEIGHT	URINE			CREATINE INJECTED	CREATINE RECOVERED IN URINE
		Volume	Creatinine	Creatine		
	<i>kgms.</i>	<i>cc.</i>	<i>mgms.</i>	<i>mgms.</i>	<i>mgms.</i>	<i>per cent</i>
1	1.60	280	57	0		
2	1.61	270	58	0		
3	1.65	230	57	0		
4	1.67	235	58	0		
5	1.69	250	54	0		
6	1.68	210	53	0		
7	1.63	285	60	0		
8	1.63	260	60	0		
Average.....			57			
9	1.65	260	60	244	328	74
10	1.66	250	66	237	306	77
11	1.68	285	67	35	131	27
12	1.65	225	70	26	109	24
13	1.67	230	61	30	77	39
Average.....			65	114	190	60
14	1.66	235	68	0		
15	1.69	260	61	0		

Female albino, received 350 grams carrots daily.

On days 9, 10, 11, the creatine was given in 3, 4 and 2 doses respectively, in the course of 4 to 6 hrs.

Skinned and eviscerated carcass—870 grams.

TABLE IV.
Rabbit 49—Injection of creatine.

DAY	BODY WEIGHT	URINE			CREATINE INJECTED	CREATINE RECOVERED IN URINE
		Volume	Creatinine	Creatine		
	<i>kgms.</i>	<i>cc.</i>	<i>mgms.</i>	<i>mgms.</i>	<i>mgms.</i>	<i>per cent</i>
1	1.77	210	54	0		
2	1.82	240	54	0		
3	1.79	155	54	0		
4	1.74	255	51	0		
Average.....			53			
5		295	60	7	68	18
6	1.70	295	54	23	68	
7	1.68	255	59	5	68	
8	1.70	295	65	10	68	
9	1.70	295	56	14	68	
10	1.70	225	60	16	77	31
11		215	60	11	85	
12	1.78	250	65	41	120	
13	1.70	240	54	31	85	
14	1.69	225	61	34	134	
15	1.70	260	54	55	134	
16	1.72	225	53	16	45	
Average.....			58	22	85	26
17	1.74	260	52	0		
18	1.74	275	52	0		
19	1.75	275	50	0		

Grey female, received 350 grams carrots daily.
 Skinned and eviscerated carcass—964 grams.

TABLE V.
Rabbit 56—Injection of creatine.

DAY	BODY WEIGHT	URINE			CREATINE INJECTED	CREATINE RECOVERED IN URINE
		Volume	Creatinine	Creatine		
	kgms.	cc.	mgms.	mgms.	mgms.	per cent
1	1.87	230	64	0		
2	1.88	285	65	0		
3	1.88	305	65	0		
4	1.88	315	69	0		
5	1.85	310	71	0		
Average.....			67			
6	1.85	270	69	32	89	39
7	1.85	280	69	35	89	
8	1.85	330	81	75	179	42
9	1.81	280	70	5	0	
10	1.80	350	70	24	134	30
11	1.79	315	71	52	134	
12	1.80	285	63	44	134	
13	1.81	335	78	13	89	21
14	1.83	290	54	24	89	
15	1.82	290	55	30	116	26
Average.....			68	33	105	32
16	1.83	325	66	0		
17	1.80	285	61	0		
18	1.80	305	70	0		

Brown male, received 350 grams carrots daily.

On the 8th day the creatine was given in two equal doses at an interval of 6 hours.

Skinned and eviscerated carcass—1025 grams.

TABLE VI.
Rabbit 59—Injection of creatine.

DAYS	BODY WEIGHT	URINE			CREATINE INJECTED	CREATINE RECOVERED IN URINE
		Volume	Creatinine	Creatine		
	<i>kgms.</i>	<i>cc.</i>	<i>mgms.</i>	<i>mgms.</i>	<i>mgms.</i>	<i>per cent</i>
1	2.07	295	80	0		
2	2.07	190	78	0		
3	2.12	160	72	0		
4	2.12	240	72	0		
5	2.07	200	78	0		
Average			76			
6	2.10	265	90	38	90	52
7	2.04	250	74	39	90	
8	2.01	280	69	62	90	
9	2.00	305	80	88	135	67
10	2.00	295	68	95	135	
11	1.98	185	70	94	135	
12	2.06	280	81	133	181	75
13	2.00	350	80	146	181	
14	1.98	330	77	128	181	
Average			77	91	135	67
15	1.98	335	71	0		
16	1.95	315	74	0		
17	1.95	315	80	0		
18	1.95	280	67	0		

Black female, first seven days fed 350 grams carrots and 400 grams on remaining days of experiment.

Skinned and eviscerated carcass—1072 grams.

TABLE VII.
Injection of creatine.

DAY	BODY WEIGHT	URINE			CREATINE INJECTED	CREATINE RECOVERED IN URINE
		Volume	Creatinine	Creatine		

Rabbit 71.

	kgms.	cc.	mgms.	mgms.	mgms.	per cent
1	2.61	180	108	0		
2	2.58	85	109	0		
3	2.56	115	109	0		
4	2.63	125	108	0		
5	2.63	150	107	0		
Average			108	0		
6	2.62	185	133	50	100	34
7	2.57	100	102	30	100	
8	2.57	80	108	23	100	
9	2.55	175	123	135	200	63
10	2.51	200	131	125	200	
11	2.52	140	107	110	200	
Average			117	79	150	53
12	2.50	180	108	7		
13	2.50	205	129	0		

Rabbit 72.

	kgms.	cc.	mgms.	mgms.	mgms.	per cent
1	2.43	230	111	0		
2	2.44	50	108	0		
3	2.48	90	111	0		
4	2.48	190	112	0		
5	2.45	270	118	0		
Average			112	0		
6	2.45	240	128	32	100	30
7	2.43	275	141	20	100	
8	2.40	220	131	38	100	
9	2.43	250	122	116	200	56
10	2.40	290	133	103	200	
11	2.48	230	128	118	200	
Average			130	71	150	47
12	2.40	255	122	0		
13	2.40	270	119	0		

Both animals were males. Rabbit 71 ate 180-350 grams carrots daily; rabbit 72, 350 grams carrots daily.

On days 9, 10, 11 in both experiments the creatine was given in two doses at intervals of 2-4 hours.

TABLE VIII.
Rabbit 73—Injection of creatine.

DAY	BODY WEIGHT	URINE			CREATINE INJECTED	CREATINE RECOVERED IN URINE
		Volume	Creatinine	Creatine		
	<i>kgms.</i>	<i>cc.</i>	<i>mgms.</i>	<i>mgms.</i>	<i>mgms.</i>	<i>per cent</i>
1	1.66	125	61	0		
2	1.65	110	61	0		
3	1.61	40	56	6		
4	1.67	80	60	10		
5	1.68	125	61	7		
Average			60	5		
6	1.65	180	67	74	100	
7	1.65	180	75	120	100	
8	1.65	150	65	101	100	
9	1.66	200	66	100	100	
10	1.67	235	62	85	100	
11	1.67	230	66	65	100	
12	1.66	220	61	56	100	
Average			66	86	100	81

Female albino, ate 140-350 grams carrots daily.

The elimination of small amounts of creatine in the first period suggests the possibility that growth had not been completed.

Skinned and eviscerated carcass—847 grams.

TABLE IX.

Rabbit 57—Carbohydrate feeding; Creatine injection.

DAYS	BODY WEIGHT	DIET		DAILY OR AVERAGE DAILY URINARY DATA			CREATINE INJECTED	CREATINE RECOVERED IN URINE
		Sucrose	Water	Total N	Creatinine	Creatine		
	kgms.	grams	cc.	gram	mgms.	mgms.	mgms.	per cent
1-7	1.92	22	80	0.44	63	8		
8-12	1.57	20	60	0.41	69	7		
13	1.39	20	70	0.23	68	40	90	33
14		20	70				90	
15		20	70				90	
16		20	60				90	
17		20	50				90	
18		20	50				90	
19		20	50				90	
20	1.34	20	50	0.30	76	56	90	
21		20	50	0.20	81	65	90	
22		20	50	0.22	56	39	90	
23	1.38	20	50	0.40	87	127	90	
20-23 (Average)					75	72	90	60
24		20	50	0.29	68	38	0	
25		20	50	0.26	65	20	0	
26		20	50	0.22	51	13	0	
24-26 (Average)					61	24		
27	1.24	20	50	0.30	66	67	90	
28-29 (Average)	1.24	20	50	0.16	42	51	45 (90)	
27-29 (Average)					50	56	60	60

White and black female. From 12th to 17th day diarrhoea was present although by frequently compressing the bladder, good urine samples were obtained. After the 17th day no diarrhoea was observed. On the 29th day, the animal still appeared to be in good condition, but it was thought best to kill it so as to avoid the acute changes immediately preceding death.

Skinned and eviscerated carcass—687 grams.

TABLE X.
Rabbit 58—Injection of creatinine.

DAYS	BODY WEIGHT	URINE			CREATININE INJECTED	CREATININE RECOVERED IN URINE
		Volume	Creatinine	Creatine		
	<i>kgs.</i>	<i>cc.</i>	<i>mgms.</i>	<i>mgms.</i>	<i>mgms.</i>	<i>per cent</i>
1	1.79	355	68	0		
2	1.76	310	66	0		
3		250	67	0		
4	1.76	335	69	0		
5	1.71	290	67	0		
6	1.70	315	69	0		
7	1.79	260	64	0		
Average.....			67			
8	1.68	80	134	0	83	
9	1.58	275	131	0	83	
10	1.65	275	130	0	83	
11	1.68	290	126	0	83	
12	1.67	325	142	0	83	
13	1.67	220	107	0	83	
14	1.68	295	162	0	83	
15	1.66	265	126	0	100	
16	1.68	325	147	0	75	
Average.....			134		84	82
17	1.65	250	54	0		
18	1.68	265	64	0		
19	1.68	260	65	0		

Brown male, ate 350 grams carrots daily. On the 8th day, through an oversight, the animal was not fed, which accounts for the low volume of urine on this day.

TABLE XI.
Rabbit 62—Injection of creatinine.

DAY	BODY WEIGHT	URINE			CREATININE INJECTED	CREATININE RECOVERED IN URINE
		Volume	Creatinine	Creatine		
	<i>kgms.</i>	<i>cc.</i>	<i>mgms.</i>	<i>mgms.</i>	<i>mgms.</i>	<i>per cent</i>
1	1.70	240	64	0		
2	1.73	230	62	0		
3	1.79	240	66	0		
4	1.82	260	63	0		
Average.....			64			
5	1.85	320	146	0	83	84
6	1.80	340	130	0	83	
7	1.66	250	137	0	83	
8	1.66	255	157	0	124	
9	1.66	260	184	0	165	75
10	1.66	305	192	0	165	
Average.....			159		117	80
11	1.64	280	57	0		
12	1.65	230	59	0		
13	1.66	265	59	0		
14	1.65	195	59	0		

Black and white female, ate 350 grams carrots daily.
 Skinned and eviscerated carcass—847 grams.

TABLE XII.
Rabbit 74—Injection of creatinine.

DAYS	BODY WEIGHT	URINE			CREATININE INJECTED	CREATININE RECOVERED IN URINE
		Volume	Creatinine	Creatine		
	<i>kgms.</i>	<i>cc.</i>	<i>mgms.</i>	<i>mgms.</i>	<i>mgms.</i>	<i>per cent</i>
1	1.95	235	88	0		
2	1.96	210	88	0		
3	1.97	175	88	0		
4	1.91	65	88	0		
5	1.92	165	88	0		
Average.....			88			
6	1.95	265	174	0	100	71
7	1.94	260	152	0	100	
8	1.96	320	152	0	100	
9	1.91	290	257	0	200	80
10	1.91	285	243	0	200	
11	1.90	295	240	0	200	
Average.....			203		150	77

Male rabbit; ate 350 grams carrots daily.

Skinned and eviscerated carcass—1084 grams.

THE FATE OF PROTEIN DIGESTION PRODUCTS IN THE BODY.

II. DETERMINATION OF AMINO NITROGEN IN THE TISSUES.

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The methods will first be described. In the latter part of the paper some particular points will be discussed in detail.

I. *Method for closest absolute results.* The sample of tissue, which may weigh from 5 to 30 grams, is immediately after excision weighed to within 0.01 gram, covered with boiling hot water, to which 1 cc. of 50 per cent acetic acid per liter is added, and heated on the water bath until the proteins are completely coagulated. In case the piece is a large one it is cut into several with a pair of scissors, in order that it may heat through more readily. Coagulation is complete in twenty or thirty minutes. The pieces are then lifted out of the water with forceps or crucible tongs, minced fine with a food macerator, and returned to the same water. The heating is continued for about ten minutes, with occasional stirring, and the supernatant liquid is then decanted through a filter of glass wool. The tissue pieces are covered with a fresh portion of boiling acidified water, using 5 or 10 cc. for each gram of tissue, and the extraction repeated for five or ten minutes, when the solution is decanted through the same filter. The use of 4 or 5 successive portions of hot water in this manner insures complete extraction of the amino-acids in the tissues. The extracts are transferred to a 1-liter, double-necked distilling flask, and concentrated under diminished pressure to about 20 cc. The distillation may be run as rapidly as is possible without loss of solution by foaming. The concentrated solution is transferred with a minimum amount of water to an Erlenmeyer flask, and mixed with 9 to 10 volumes of 95 per cent ethyl alcohol, or half that amount of absolute

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ethyl or methyl alcohol. The alcohol precipitates a small amount of protein which is not coagulable by heat. The solution is allowed to stand over night to complete precipitation. It is then filtered through a folded filter, washed with 80 per cent alcohol, and returned to the distilling flask. A few drops of phenolphthalein solution are added, and enough 25 per cent sodium hydrate solution, drop by drop, to render the solution alkaline. It is then concentrated under diminished pressure to drive off both ammonia and alcohol. If the receiving flask is provided with a guard flask, as illustrated in this *Journal*, x, p. 21, 1911, containing $\frac{N}{50}$ acid, the ammonia can be determined. In the tissues of the dog it amounts to about 10 mg. per 100 grams of fresh tissue. When the volume of the concentrating solution has been reduced to 10 or 20 cc. the distillation is interrupted, and enough 50 per cent acetic acid is added to acidify the solution. About 50 cc. of water are also added, in order to insure that the last traces of alcohol shall be driven off, and the concentration is continued. In case the amount of tissue analyzed exceeds 10 grams, the distillation may be continued in the same 1 liter flask, but if the sample is smaller the solution should now be transferred to a flask of 300 to 500 cc. The extracts are concentrated to a few cc., and are then transferred, with several small portions of water, to a measuring flask. The size of the latter depends on the weight of tissues taken for analysis. When the amount is under 10 grams we use a 10 cc. flask to hold the final solution; when over 10 grams have been taken, a 25 cc. flask is used.

The solution is now ready for the amino determination. One can take a 10 cc. portion and use the larger amino apparatus,¹ but we have found it in general somewhat more convenient to take 2 cc. portions and employ the micro-apparatus.² The length of time which the reacting solution should be shaken in order to drive off all the amino-acid nitrogen depends somewhat on the temperature. When the latter is 15-20° the time should be five to four minutes; for 20-25° it is three minutes; for 25-30°, two and a half to two minutes. It is preferable that the solution should be shaken vigorously with a motor and the time kept down to these limits, for the sake not only of rapidity, but of accuracy. The

¹ This *Journal*, xii, p. 275, 1912.

² *Ibid.*, xvi, p. 121, 1913.

reason for this is, that, even after removal of the ammonia, the extracts contain small amounts of urea and other substances which belong to the class of slowly reacting amines, and are therefore not α -amino-acids. The amount of nitrogen which these amines give off in the time required for amino-acids to react completely is small, but if the reaction were allowed to run for an indefinite length of time instead of being kept to a definite minimum the error might be both large and variable. The correction for the amount of nitrogen given off by these amines while the amino-acids are being decomposed is ascertained in the same manner as in blood analysis,³ by continuing the reaction, after the gas from the α -amino-acids has all been driven off, for a length of time equal to that utilized in decomposing the amino-acids (two to five minutes according to the temperature), and then measuring separately the gas evolved during this second reaction period. The correction found is fairly constant at about 6 per cent of the total amino nitrogen obtained.

II. *Simpler method for accurate comparable results.* As the correction for amines other than α -amino-acids is small and fairly constant, it can be left out without decreasing appreciably the constancy of results, or affecting the determination of differences in amino-acid content. The effect of the ammonia present is also small and practically constant. Consequently when, as in most physiological work, differences rather than strictly absolute results are desired, one can simplify the above outlined method by leaving out the determination of the correction and the removal of the ammonia. The results agree as well as when the ammonia is removed and other amines are corrected for, but are about 10 per cent higher. One must, under any conditions, however, accurately control the time of the reaction in the amino apparatus. In our own experimental work we have always removed the ammonia. After determining the correction for amines other than amino-acids in experiments with about 20 dogs, however, without finding that it varied appreciably under any conditions or added to the significance of the results, we ceased to utilize it.

The *accuracy* of the determination is limited, not so much by sources of error in the method, as by the fact that one cannot obtain absolutely homogeneous samples of tissue. Duplicate amino

³ Van Slyke and Meyer: *This Journal*, xii, p. 402, 1912.

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determinations on the same solution of tissue extract usually agree as closely as one can read the volume of nitrogen gas in the burette. The error in the final amino determination is practically negligible. We have also convinced ourselves that there is very little error connected with the processes of extracting the tissues and concentrating the extracts in the manner outlined above. If one analyzes tissue which has been dried and pulverized, so that different samples of material have the same composition, results with different portions do not vary more than 1 or at most 2 mg. of amino nitrogen per 100 grams of fresh tissue, the amino figure for the latter usually falling between 40 and 80 mg. per 100 grams. When, however, different portions of an organ, such as the lobes of the liver, are taken as duplicate samples or when symmetrically placed organs, such as the right and left gracilis muscles or the two kidneys, are used, one must expect variations up to 10 per cent of the amount of amino nitrogen determined.

The two concentrations under diminished pressure involved in the analysis cannot be replaced by concentrations on the water bath. During the latter the extracts darken, and part of the amino nitrogen disappears. The effect on results is especially marked with liver extracts. If the latter are concentrated to dryness and then taken up in water a black solution results, which may show less than half the amino nitrogen originally in the extract.

The chief problems of the analysis were the complete extraction of the amino-acids from the tissues, and the removal of the proteins from the extract, both operations being necessarily performed under conditions which result in neither loss of amino-acids nor their formation by hydrolysis of the protein present. Folín and Denis in their methods for tissue analyses met these conditions by extracting the tissues with cold methyl alcohol. This method was not suitable for our purposes, however, as alcohol extracts the lipoids, and they may settle out of the final water solution in such masses that they mechanically hinder an accurate amino determination. Extraction with hot water is much more rapid than with alcohol, and the extract contains a relatively small amount of lipoids. It contains an appreciable amount of protein, however. For its removal we found the above described alcohol treatment the most satisfactory general method, although it makes two vacuum concentrations necessary, when one would be suffi-

cient if the proteins could be satisfactorily thrown out of the water extract by one of the usual precipitants. We tried a number of these, and found that metaphosphoric acid in particular gave results with muscle extracts which were satisfactory and agreed with those by the alcohol method, but that in liver extracts, presumably because of the glycogen present, it failed to precipitate the protein satisfactorily.

The only apparent objection to the hot water extraction is, that it might increase the amino nitrogen by hydrolysis of some of the proteins present. That such hydrolysis does not occur appears from the following experiment.

Muscles fresh from the thigh of a dog were cut into pieces with scissors and dried over sulphuric acid at a pressure of 0.2 mm. The dried muscles were pulverized and thoroughly mixed. Samples of 3 grams each were weighed out, placed in flasks with 100 cc. each of hot water, and allowed to digest at 100° for periods of five minutes, thirty minutes, and one, two,* and three hours respectively. At the end of the period of digestion the water was decanted through a glass wool filter, and the residue in the flask washed with three 75 cc.-portions of water at 100°, each portion being allowed to remain five minutes on the muscle shreds. In case the action of hot water on the tissues produces a sufficiently rapid hydrolysis to become a factor in disturbing the accuracy of results it should make itself apparent by increased amino nitrogen content in the samples which were digested longest. That this was not the case is shown in the table below. The water extracts were concentrated and precipitated with alcohol, and, after the alcohol had been driven off, the entire solution was used for determination of amino nitrogen in the larger apparatus.

TABLE I.

PERIOD OF DIGESTION WITH HOT WATER	CC. NITROGEN GAS AT 20", 760 MM.
5 minutes.....	11.4
30 minutes.....	11.4
1 hour.....	11.5
2 hours.....	11.2
3 hours.....	11.2

The results also indicate the accuracy of the methods, from the initial extraction to the final determination, when the samples are taken from homogeneous material.

The results of the following experiment exemplify some points of interest.

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Fresh muscle of 123 grams' weight was extracted with hot water and the water extract brought to 100 cc. The 100 cc. were divided into five portions of 20 cc. each. The non-coagulable protein was left in No. 1. From the others it was removed by the means indicated in the table. The solutions were freed from ammonia and eventually all brought to 25 cc., of which 2 cc. portions were used for duplicate determinations of amino nitrogen. Ten cc. portions were, furthermore, mixed with 10 cc. each of concentrated hydrochloric acid and heated twenty-four hours to completely hydrolyze the proteins and intermediate products present.⁴ The hydrochloric acid was driven off as thoroughly as possible by concentrating in vacuum, and the ammonia boiled off in vacuum with calcium hydrate. The residual solutions were diluted to their original volume of 10 cc., and 2 cc. portions taken for amino determinations.

The methyl and ethyl alcohols used were "absolute;" the zinc chloride solution contained 5 grams of the chloride dissolved in 100 cc. of 80 per cent ethyl alcohol.

TABLE II.

NO.	METHOD OF PRECIPITATING PROTEINS IN 20 CC. WATER EXTRACT	AMINO N PER 100 GRAMS MUSCLE		PEPTIDE BOUND N
		Free	After hydroly- sis of extract	
1	Not precipitated.....	89	224	135
2	100 cc. methyl alcohol.....	68	100	32
3	100 cc. methyl alcohol+2 cc. ZnCl ₂ solution.....	55	89	33
4	100 cc. ethyl alcohol.....	65	98	33
5	100 cc. ethyl alcohol+2 cc. ZnCl ₂ solution.....	58	85	27

Alcohol alone added to the water extracts precipitates chiefly proteins and higher intermediate products. The free amino nitrogen precipitated by methyl alcohol was $89 - 68 = 21$ mg. The peptide bound nitrogen was $135 - 32 = 103$ mg. The ratio (free NH_2 : peptide bound NH_2) in the precipitate was, therefore, approximately 1 : 5. The ratio in animal proteins is usually about 1 : 12 (the animal proteins containing about 5 per cent of their nitrogen as free amino nitrogen, about 60 per cent more being set free by hydrolysis). The ratio 1 : 5 shows that the alcohol precipitate consisted, in part at least, of intermediate products, but that it could have contained little or no free amino-acid nitrogen. Similar

⁴ Conditions for Complete Hydrolysis of Proteins, *This Journal*, xii, p. 295, 1912.

results are found with ethyl alcohol as a precipitant. It removes proteins and intermediate products, but leaves the amino-acids.

The action of zinc chloride, which when added to the alcoholic mixture precipitates some substances that alcohol alone does not, is different. It apparently precipitates some free amino-acids, without much affecting the intermediate products. The decrease in free amino nitrogen caused by adding the action of zinc chloride to that of alcohol (compare No. 2 with No. 3 and No. 4 with No. 5) is greater than that of the peptide bound nitrogen. One can also obtain a precipitate with zinc chloride and alcohol in a slightly acid solution of the products of complete acid hydrolysis of casein, and the precipitate removes part of the amino nitrogen from the solution. It would seem, therefore, that zinc chloride in alcoholic solution precipitates some amino-acid or acids. We have not further investigated the point, as it is of minor interest, but because of the above results and others like them we have not utilized zinc

TABLE III.

SAMPLE	WEIGHT OF SAMPLE	N HCl TO NEUTRALIZE AMMONIA	AMMONIA N PER 100 GM. TISSUE	CC. N ₂ FROM 10 CC. OF FINAL 25 CC. SOLUTION (Correction for other amines placed below each result and subtracted)	TEMPERATURE	PRESSURE	DURATION OF REACTION WITH HNO ₃	MG. AMINO-ACID N PER 100 GM. TISSUE	
								Corrected for other amines	Uncorrected
Left triceps muscle.....	14.98	5.1	10	6.75	21	758	4	60	64
				0.40			4		
				6.35					
Right triceps muscle.....	20.37	7.9	11	8.70	23	760	4	56	60
				0.60			4		
				8.10					
Liver, lobe 1.	11.81	3.9	9	6.55	22	758	4	72	76
				0.40			4		
				6.15					
Liver, lobe 2.	19.62	7.2	10	10.60	21	758	4	73	77
				0.50			4		
				10.10					

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chloride as a precipitant. It would not be likely to affect the significance of comparative results, however, like those of Folin and Denis, because the proportion of amino nitrogen precipitated is quite constant.

The results in table III, taken from data in connection with Dog 17, serve as examples of the order of magnitude of the figures obtained, the volumes of gas measured, etc. The determinations were performed according to the "absolute" method, the ammonia being removed, and the correction for the amines, other than α -amino-acids, being determined.

The following table, from analyses of another dog, gives some typical figures.

TABLE IV.

TISSUE	FREE AMINO N IN WATER EXTRACT, BEFORE REMOVAL OF PROTEINS NOT COAGULATED BY HEAT	AMINO N IN EXTRACT AFTER PRECIPITATION OF PROTEINS WITH ALCOHOL		
		Free NH_2	NH_2 after complete hydrolysis with HCl	Peptide bound N (NH_2 freed by hydrolysis)
Gracilis				
muscle.....	50	38	61	23
Heart.....	50	37	61	24
Liver.....	75	61	89	28
Spleen.....	64	53	68	15
Kidney.....	73	65	77	12
Stomach.....	41	32	53	21
Duodenum...	66	50	60	10

The results in the second and third columns show that the amino nitrogen found in the extract after treatment with alcohol (second column) comes chiefly from free amino-acids. In the case of even the simplest peptides, the dipeptides, the free amino nitrogen is doubled by hydrolysis, and in the primary albumoses it is increased about eight times. Here the increase is only 20 to 50 per cent. A mixture of amino-acids and albumoses such that 93 per cent of the free amino nitrogen belongs to the amino-acids and only 7 per cent to the albumoses, would give an increase of 50 per cent in the amino nitrogen on hydrolysis of the albumoses. Such a relation apparently exists in the tissue extracts as prepared for analysis; for even after the alcohol treatment they show the presence of traces of protein or higher intermediate products when

tested for biuret with the precautions given by van Norman.⁵ The relations between the free amino nitrogen and the peptide bound nitrogen given in the above table are typical. A much larger proportion of peptide bound nitrogen is never found. One can, therefore, depend upon figures for amino nitrogen in the tissues obtained by the above method as representing with a fairly close degree of approximation the simple amino-acids.

SUMMARY.

The amino-acids are extracted from the tissues with hot water. Uncoagulated proteins in the extract are precipitated by alcohol. Alcohol and the slight amount of ammonia present in the extract are removed by concentration in vacuum, and the amino nitrogen in the residue is determined by the nitrous acid method. The rapidity with which the amino nitrogen reacts with nitrous acid, and the relatively small increase which it shows as the result of hydrolysis of the extract with hydrochloric acid, indicate that the amino nitrogen determined by the method outlined represents approximately the *free α -amino-acids*. Only a few per cent of the amino nitrogen appears due to proteins or their intermediate products, and to amines not of protein origin. The correction for the latter can, when desirable, be readily determined.

⁵ *Biochem. Journ.*, iv, p. 127, 1909.



THE FATE OF PROTEIN DIGESTION PRODUCTS IN THE BODY.

III. THE ABSORPTION OF AMINO-ACIDS FROM THE BLOOD BY THE TISSUES.¹

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In our preliminary communication² we mentioned only the most recent articles on the fate of the products of protein digestion. In order that our work may appear in its proper relation to that which has previously been done in the field, it appears desirable to review the latter more fully before proceeding with the report of our own results.

The contributions on the subject appear to be most readily grouped around the different explanations of the fate of ingested protein which have served as working hypotheses. These hypotheses may be formulated as follows:

1. The ingested proteins are absorbed and incorporated into the body without undergoing any marked chemical change.
2. The food proteins are first hydrolyzed in the alimentary tract; the products of digestive hydrolysis are then absorbed into the blood and carried to the tissues.
3. The products are deaminized in the wall of the intestine before entering the circulation.
4. The products are synthesized into serum protein before entering the circulation. The serum proteins thus formed serve as nourishment for the tissues in general.

We will take up these hypotheses in their order, reviewing the work which appears most important in supporting or antagonizing each.

¹ The results in this paper were reported in abstract at the meeting of the Soc. Exper. Biol. and Med., Dec. 18, 1912. *Proceedings*, x, p. 38.

² This *Journal*, xii, p. 399, 1912.

1. The simplest, and apparently the earliest theory concerning the manner in which the proteins of the food reach the tissues and are incorporated into them, is that the proteins are absorbed, with little or no chemical change, directly into the circulation, from which they are taken by the tissues and incorporated into their substance. In support of this view the fact was demonstrated that unchanged proteins could be made to pass directly from the alimentary canal into the blood.³ Egg albumin can even be absorbed in such amounts that it appears in the urine.

More thorough investigation has shown decisively, however, that the absorption of unchanged proteins is an abnormal process. From a knowledge of the proteolytic powers of the digestive tract attained by the famous experiments of Spallanzani and Beaumont with the gastric juice, the discovery of trypsin by Kühne,⁴ and the work of many later investigators, including Cohnheim,⁵ the discoverer of erepsin, one is justified in stating that the more deeply the processes in the alimentary tract have been studied the more thorough has the breakdown of proteins in normal digestion been found. Cohnheim's work on this point is particularly important. He fed meat to a dog with a duodenal fistula which permitted isolation of the products of digestion after they had passed the stomach and part of the intestine. The partially digested products obtained from the fistula were treated with erepsin for twenty-four hours, the action of this enzyme thus following, as in normal digestion, that of the gastric and pancreatic juices. The meat proteins were hydrolyzed so completely that all the arginine was free⁶ and the nitrogen precipitable by phosphotungstic acid could not be further decreased by boiling with sulphuric acid.⁷ No evidence of the presence of peptides or intermediate products could be found. It appears probable, therefore, that normal digestion proceeds in the intestinal lumen and wall until most, if not all, of the proteolytic products are reduced to the stage of free amino-acids. Abderhalden and his co-workers have isolated nearly all of the known

³ For references see article by Cohnheim: *Zeitschr. f. physiol. Chem.*, xxxv, p. 397.

⁴ *Virchow's Archiv*, xxxix, p. 155.

⁵ *Zeitschr. f. physiol. Chem.*, xxxiii, p. 451.

⁶ *Ibid.*, li, p. 415, 1907.

⁷ *Ibid.*, xlix, p. 64.

amino-acids from intestinal contents, and have furthermore shown that protein which has been digested completely into amino-acids is as efficient as intact protein in maintaining the nitrogenous equilibrium and even the growth of dogs.⁸ Abderhalden has also found positive evidence that intact protein is not normally absorbed into the circulation.⁹ Injection of protein into the circulation results in the development of a proteolytic enzyme in the blood capable of hydrolyzing the injected protein. The fact that the normal blood is free from such enzymes shows that it does not absorb undigested proteins. Furthermore, the fact that it is difficult or impossible to develop an anaphylactic state by protein feeding is proof against absorption of proteins as such, even in small amounts. Also, attempts to find evidence of food proteins in the blood by the precipitin reaction have given negative results.¹⁰

2. The absorption of intact proteins being an untenable hypothesis, the simplest alternative explanation is that the "peptone," or the mixture of digestive products, is absorbed directly into the blood and conveyed to the tissues.¹¹ That this mode is not impossible is indicated by recent work of Buglia.¹² He found that completely digested flesh in amounts equivalent to a day's protein requirement could be injected intravenously into dogs without injurious effect if several hours were taken for the injection, so that the rate of entrance of the products was similar to the rate of absorption in normal digestion. The injected products were mostly metabolized and excreted as urea.

That during the actual protein digestion, however, the final hydrolytic products enter directly into the circulation without undergoing chemical change while passing the intestinal wall, remained uncertain because of the many failures to demonstrate these products in the normal blood. For several decades it has been investigated for the presence of peptone with negative results.¹³ When

⁸ *Synthese der Zellbausteine.*

⁹ *Schutzfermente.*

¹⁰ Debré and Porak: *Journ. phys. et path. gén.*, xiv, p. 1019.

¹¹ A clear presentation of this view was given in 1905 by Folin: A Theory of Protein Metabolism, *Amer. Journ. of Physiol.*, xiii, p. 117. A thorough discussion of work up to 1912 on this subject is given by Cathcart: *Physiology of Protein Metabolism*, chapter on protein regeneration.

¹² *Zeitschr. f. Biol.*, lviii, p. 162, 1912.

¹³ Abderhalden and Oppenheimer: *Zeitschr. f. physiol. Chem.*, xlii, p. 155, 1904; Howell: *Amer. Journ. of Physiol.*, xvii, p. 273, 1906.

the importance of amino-acids as the end products of digestion became appreciated these also were sought, but the most careful work failed to result in the isolation of a single amino-acid from normal blood, even during the height of digestion.¹⁴

Abderhalden, Gigon, and London were able, it is true, after injecting alanine into the stomach of a dog, to isolate that amino-acid from the blood and urine as the naphthylsulpho compound.¹⁵ Results likewise indicating the ability of protein digestion products to pass from the intestine into the circulation were obtained by Cathcart and Leathes.¹⁶ They found an increase in the "residual nitrogen" (nitrogen left after subtraction of the urea and removal of the protein precipitable by heat and by tannic acid) following the absorption of peptone injected into a loop of the intestine of a dog. An increase in the non-protein nitrogen of the liver was also noted. Results on the blood similar to those of Cathcart and Leathes have recently been obtained with a refined technique by Folin and Denis¹⁷ after injection of amino-acids into the intestines of cats. These authors have also determined an increase in the non-protein nitrogen of the muscles following the absorption of amino-acids from the intestine. These results, however, can hardly claim the same degree of finality as those of Folin and Denis on the question of deaminization and urea formation to be shortly discussed. The interpretation of changes in non-protein nitrogen as changes in amino-acid nitrogen is arbitrary, as there was no evidence concerning the chemical nature of the nitrogen in which the changes were noted, aside from the proof that it was not in the form of urea or ammonia.

The fact that amino-acids *can* enter the blood from the alimentary canal when the latter has been flooded with them, although clearly demonstrated by Abderhalden, Gigon, and London, did not carry with it the proof that absorption of unchanged amino-acids into the circulation occurs during their gradual liberation in digestion, nor was it regarded as such proof by these authors. Egg albumin can also be made to enter the blood and even appear in the urine when the alimentary canal is flooded with it under properly

¹⁴ Abderhalden: *Synthese der Zellbausteine*.

¹⁵ *Zeitschr. f. physiol. Chem.*, liii, p. 113, 1907.

¹⁶ *Journ. of Physiol.*, xxxiii, p. 463, 1906.

¹⁷ *This Journal*, xi, p. 87, 1912.

chosen conditions. Proof of the presence of amino-acids in the blood under normal conditions none of the investigators in the field was able to accomplish, despite the application of all the methods known to modern chemistry for the isolation of these substances. Investigation of the residual nitrogen of the blood indicated the possibility of the absorption of amino-acids or peptides. The failure to ascertain the chemical nature of the fraction of residual nitrogen to which the changes noted were due, however, made the results inconclusive, as those based on residual nitrogen determinations noted in the preceding paragraph would also have been, had they not been confirmed by the more definite findings of Abderhalden, Gigon, and London. Hohlweg and Meyer¹⁸ found in the serum of fasting dogs an average residual nitrogen of 7 mg. per 100 cc. In digesting animals the average figure was 13 mg. As the amounts were so small, however, the individual fluctuations considerable, and in particular as there was no evidence of the chemical nature of the residual nitrogen, the results could not be regarded as decisive. Howell attacked the problem¹⁹ with the aid of naphthylsulfochloride, which had been introduced by Fischer and Bergell as a precipitant for amino-acids. He obtained a precipitate in the dialysate of the serum, and the bulk precipitated was observed to be larger in the serum of fed dogs than in that of fasting animals. The precipitate was an oil, however, which could neither be identified chemically nor measured quantitatively. For this reason, notwithstanding the interesting possibilities indicated by the results, it could not be stated with certainty what proportion, if any, of the precipitate was due to amino-acids. Cohnheim, working with the alimentary canal of the octopus under conditions which to some extent simulated the natural, succeeded in separating crystalline amino-acids from the blood.²⁰ The tract under normal conditions practically floats in the blood of the animal. Cohnheim removed the tract with the digestive glands attached, filled it with peptone solution, and floated it for twenty hours in blood through which oxygen was passed. The organs remained alive during the experiment. At the end of the latter the residual nitrogen of the blood, which is ordinarily almost nil, was found

¹⁸ *Hofmeister's Beiträge*, xi, p. 381, 1908.

¹⁹ *Amer. Journ. of Physiol.*, xvii, p. 273, 1906.

²⁰ *Zeitschr. f. physiol. Chem.*, xxxv, p. 396, 1902.

to be greatly increased. No peptone could be detected in it, but ammonia was determined, and leucine, tyrosine, and lysine picrate were crystallized from it, though not enough of any was obtained for analysis. Cohnheim was conservative about generalizing these results to apply to normal digestion in the higher animals, and his reserve appeared to be justified by later results which he obtained in repeating the above experiment with a vertebrate fish, *Crenilabrus pavo*. He obtained ammonia in the external blood, but no evidence of either mono- or diamino-acids, and concludes: "dass bereits beim Passieren der Darmwand die Eiweisspaltprodukte teilweise desamidiert werden und in Ammoniak und einen, zunächst unbekannten Rest zerfallen."²¹

3. In the view of the failure to obtain conclusive proof of the presence of amino-acids in the blood of the higher animals during digestion, the above results of Cohnheim suggested the possibility that the amino-acids are *deaminized while passing the intestinal wall*, the first stage of their catabolism occurring before they enter the circulation. This hypothesis derived support from earlier work of Nencki, Zaleski, Pavlov, Salaskin, and Horodynski, who found the ammonia content of the portal blood greater than that of the arterial during digestion.²² None of these results proved, however, that the greater part of the amino-acids suffers decomposition during absorption, and the deaminization hypothesis has in all events been effectively retired by recent work of Folin and Denis.²³ Using delicate quantitative methods which they had developed for the determination of ammonia and urea, they found that neither of these products appeared in increased amounts in the blood during absorption of glycocoll or alanine from a loop of the small intestine of the cat. They also showed that the ammonia of the portal blood is due largely to the products of putrefaction in the intestine.

4. The remaining explanation of the means by which the products of protein digestion reach the tissues without appearing in the blood is the antithesis of the recently demolished deaminization hypothesis. It assumes that the products in passing the intestinal wall, instead of being decomposed, are *synthesized into protein again*, and

²¹ *Zeitschr. f. physiol. Chem.*, lix, p. 239, 1900.

²² For references, see Cohnheim: *ibid.*, lix, p. 246.

²³ *This Journal*, xl, p. 161, 1912.

that the result of the synthesis is one or more of the proteins of the serum. This explains at one stroke both the failure to find amino-acids in the blood and the origin of its proteins. The explanation was founded on less positive evidence than the deaminization hypothesis, but its long life shows that it possessed the advantage of being difficult to disprove. The hypothesis was clearly formulated at least as early as 1870 by Funke:²⁴ "Da im Blute und im Chylus gar keine oder nur Spuren von Peptonen sich finden, so bleibt keine andere Annahme übrig, als die, dass die Peptone unmittelbar nach ihrer Aufsaugung, gleichviel ob dieselbe in's Blut oder Chylus oder beide stattfindet, in gewöhnliche Eiweisskörper, vielleicht in die gleiche Modification, das Serumalbumin, zurückverwandelt werden." Hoppe-Seyler localized the process of resynthesis even more definitely:²⁵ "Da nun in Magen und durch das Pankreassekret Acidalbumin und Pepton gebildet wird, so scheint auch das eine Funktion der Epithelzellen des Darmes zu sein, diese Körper in Serumalbumin und fibrinbildende Stoffe überzuführen." Evidence which might be interpreted in favor of the resynthesis theory was brought by Hofmeister,²⁶ who found that the stomach wall of a digesting dog contained peptone, which, however, disappeared rapidly when the stomach was kept for a half hour or more at 40°. Hofmeister pointed out that the disappearance could be due to either resynthesis or further digestion of the peptone. Glässner²⁷ decided the question in favor of resynthesis. He found that the disappearance of non-coagulable nitrogen was due entirely to the albumoses (fraction not precipitated by heat, but precipitated by saturation with zinc sulphate), the nitrogen in the filtrate from the albumoses, which contained the products of further digestion, remaining constant after removal of the stomachs from the animals. Embden and Knoop,²⁸ however, who repeated the experiment, with the difference that they used intestine instead of stomach, found that the decrease in albumoses was accompanied by an increase in their filtrate, and was therefore to be attributed

²⁴ *Lehrbuch der Physiologie*. Quoted by Popoff: *Zeitschr. f. Biol.*, xxv, p. 427, 1889. Popoff believed that the synthesis occurred in the lumen of the tract, before absorption.

²⁵ *Pflüger's Archiv*, vii, p. 399.

²⁶ *Zeitschr. f. physiol. Chem.*, vi, p. 69, 1882.

²⁷ *Hofmeister's Beiträge*, i, p. 329, 1902.

²⁸ *Ibid.*, iii, p. 120, 1903.

to further digestion of the albumoses rather than to their resynthesis into protein. This had been advanced by Cohnheim²⁹ as the probable explanation of Hofmeister's results, after Cohnheim had discovered the activity of the erepsin of the intestinal wall. Aside from the results of Glässner, which are opposite to those later obtained by Embden and Knoop, it appears that no positive evidence has ever been found for the hypothesis that the products of protein digestion are resynthesized in the walls of the alimentary canal into blood protein.

The real evidence, the failure to identify the products of protein digestion in the blood, has been purely negative. This evidence, we believe we are justified in stating, has, even if one cannot admit the conclusiveness of the significant work of Howell, been decisively eliminated by the results published by us a year ago.³⁰ Using the nitrous acid method for determination of amino groups under precautions which render it specific for α -amino-acids, we found that the latter are always present in the blood of dogs, the amount of amino-acid nitrogen being 3 to 5 mg. per 100 cc. of blood in animals after twenty-four hours fasting. After a meal of meat the figure rose to 10–11 mg. in the same animals. The results not only dispelled the negative evidence on which, because of lack of sufficiently sensitive methods, the resynthesis hypothesis had been built, but afforded positive proof that the products of protein digestion enter directly into the circulation. The amount of amino-acid nitrogen present at any one time in the blood is small, because amino-acids which enter it leave it with great rapidity. We found that intravenously injected alanine disappeared from the circulation almost as fast as it entered. A similar disappearance of injected amino-acids had shortly before been already noted by Woelfel.³¹

Immediately after our paper one on the same subject by Abderhalden and Lampé appeared.³² In their work the amino-acid nitrogen of the blood was detected by the ninhydrin color reaction, the intensity of the color developed affording comparative results.

²⁹ *Zeitschr. f. physiol. Chem.*, xxxiii, p. 451, 1901.

³⁰ *This Journal*, xii, p. 399, 1912.

³¹ *Proc. Amer. Physiol. Soc.*, Abstracts, 1911, p. 38; published in *Amer. Journ. of Physiol.*, xxix, p. xxxviii, 1912.

³² *Zeitschr. f. physiol. Chem.*, lxxxi, p. 473, 1912.

The results confirmed ours, but the authors still favor the resynthesis hypothesis, believing that the main portion of the digestion products is resynthesized into blood protein during absorption. The passage of amino-acids unchanged into the circulation during digestion they explain on the basis of the difference in composition between the proteins of the food and the blood respectively. The food proteins contain certain amino-acids in greater proportions than the blood proteins, and some of these amino-acids will necessarily be left over when the maximum amount of serum protein has been synthesized from the food. It is, according to Abderhalden and Lampé, only these superfluous amino-acids that pass unchanged into the circulation. The part of the absorbed products important for nutrition is that which enters the circulation as serum protein, and it is the serum protein, according to Abderhalden, that nourishes the tissues in general.³³ These take up the protein from the serum, hydrolyze it again into amino-acids, and from the latter reconstruct their own proteins. In regard to the ascertained facts (absorption of amino-acids directly into the circulation during digestion) there is no disagreement between Abderhalden and ourselves. The above hypothesis, however, notwithstanding the valuable work which it has stimulated, is not, it seems to us, the most probable explanation of the facts thus far at our disposal. It assumes a number of processes (synthesis of absorbed amino-acids in the intestinal wall to serum protein, utilization of serum protein as pabulum by the body cells) as yet quite undemonstrated by established facts. Moreover, the demonstrated mechanism, by which the amino-acids liberated during digestion are absorbed directly into the circulation and transferred to the tissues, is sufficient to handle these products as rapidly as they are formed; and we know at present of no ground for assuming additional and more complicated processes to provide the tissues with protein constituents. In brief, it has been found that an undetermined proportion, possibly all, of the amino-acids formed in digestion passes unchanged into the circulation; and it has not yet been shown that any of them, except such as may be altered by bacteria, are either conjugated or destroyed before entering the blood stream.

Recently a paper appeared by Rona³⁴ which, furthermore, offered

³³ *Synthese der Zellbausteine.*

³⁴ *Biochem. Zeitschr.* xlv, p. 307, 1912.

evidence that no large fraction of the amino-acids suffers chemical change while passing the intestinal wall. Surviving intestines of cats were suspended in Tyrode's salt solution and filled with solutions of amino-acids or of digested peptone, the amino nitrogen content of these solutions having been determined by the gasometric method. After several hours, during which the intestines maintained their vitality and motility, from one-half to one-third of the amino nitrogen had diffused through the intestinal walls into the Tyrode's solution. There was no decrease in the total amount of amino nitrogen present, such as would have occurred if the passage of the intestinal wall had been accompanied by a synthesis of protein. Although Rona himself did not claim that his results were conclusive, inasmuch as there was no circulation of blood through the intestines and conditions were therefore not entirely comparable to those in the living animal, absolutely negative results would hardly have been expected if the intestines normally possess the ability to synthesize protein at the rate necessary to keep pace with absorption.

From the above review it appears that the positive results of previous work on the problem before us can be condensed into the following statement: *Ingested proteins are hydrolyzed in the digestive tract setting free most, if not all, of their amino-acids. These are absorbed into the blood stream, from which they rapidly disappear as the blood circulates through the tissues.*

In the present paper we attempt to answer the question: *What becomes of the amino-acids when they vanish from the circulation?* Are they decomposed in the blood: are they at once synthesized into new protein; are they chemically incorporated into the complex molecules of the tissue proteins; or are they merely absorbed by the tissues in general, or by certain tissues in particular, without undergoing any immediate change?

EXPERIMENTAL.

Experiment 1. A male dog of 9 kg. weight, which had fasted four days, was etherized and kept with artificial respiration by the Meltzer and Auer insufflation method during the entire experiment. The bladder was washed out through a catheter, and a sample of 25 cc. of blood was drawn from the right femoral artery. The right gracilis muscle, a lobe of the liver, a short section of the

small intestine, and the right kidney were removed and coagulated for determination of amino nitrogen. The lobe of liver was isolated by means of a large clamp at the base before excision; the other samples were dissected and tied off, so that all were taken practically without loss of blood. One hundred and fifty cc. of a solution of the amino-acids obtained by hydrolysis of casein were then injected into the right femoral artery. The solution was made by boiling casein forty-eight hours with seven times its weight of 33 per cent sulphuric acid. The sulphuric acid was removed with excess barium hydrate, and the ammonia removed by concentrating the alkaline solution in vacuum. The barium was then removed with sulphuric acid, the reaction being so balanced that the filtrate from the barium sulphate gave a barely perceptible reaction for sulphate. This condition assured complete removal of the barium. The solution was concentrated in vacuum, and the slightly acid reaction was changed by adding sodium carbonate until the solution gave a barely perceptible alkaline reaction with litmus. The final solution contained 27.08 mg. of amino nitrogen per cc. It was used instead of the solution of a single amino-acid because it undoubtedly resembles more nearly the mixture of amino-acids absorbed from the intestine during digestion. The 150 cc. injected contained 4.06 grams of amino nitrogen.

The duration of the injection was thirty minutes. Half an hour after it had been finished another sample of 25 cc. of blood was drawn, the dog was killed by bleeding, and samples of the tissues again taken.

During the period following the injection 125 cc. of urine were voided through the catheter or expressed from the bladder at the end of the experiment.

The results of the analyses, which were made by the "absolute" method described in the preceding paper, are given in table I.

The blood analyses were made as described in our first paper.³⁵

The urine excreted during the experiment contained 0.738 gram of nitrogen, of which 0.463 gram, or 11 per cent of the amount injected, was amino-acid nitrogen.³⁶ Calculating the blood as 5 per cent of the weight of the animal, the increase of 41.5 mg. in the

³⁵ This *Journal*, xii, p. 402, 1912.

³⁶ For method of determining amino nitrogen in urine, see Van Slyke: *ibid.*, xvi, p. 125, 1913.

TABLE I.

TISSUE SAMPLE	MG. AMINO NITROGEN PER 100 GRAMS TISSUE	
	Before injection	Thirty minutes after injection
Liver 1.....	34	
Liver 2.....	29	
Liver 3.....		94
Liver 4.....		93
Gracilis muscle 1.....	43	
Gracilis muscle 2.....		67
Gracilis muscle 3.....		73
Kidney 1.....	45	
Kidney 2.....		106
Intestine 1.....	48	
Intestine 2.....		97
Pancreas.....		91
Spleen.....		81
Blood 1.....	3.9	
Blood 2.....		45.2
Blood 3.....		45.6

amino nitrogen indicates that 0.19 gram, or 5 per cent, of the injected amino nitrogen remained in the circulation at the end of the experiment. The intestinal juice measured, as nearly as could be estimated, 200 cc. It contained 45 mg. of amino nitrogen per 100 cc., or a total of 0.09 gram, 2 per cent of the amount injected. It is doubtful whether this small amount was due to excretion of part of the injected amino-acids into the intestine, or to traces of unabsorbed digestive products.

Summarizing the results of the experiment: *Of the amino nitrogen injected, approximately 5 per cent remained in the circulation a half an hour after the injection. Eleven per cent had been excreted in the urine. If the remaining 3.41 grams of amino nitrogen injected had been absorbed by the tissues evenly throughout the body, the average increase per 100 grams of tissue (taking the weight of tissues aside from the blood as 8.5 kg.) would have been 40 mg. The increases found were: in the muscles 27 mg.; liver, 60 mg.; kidney, 60 mg.; intestine, 50 mg. Although strictly accurate calculations are, of course, impossible, the results indicate, as closely as one can estimate from such figures, that all the amino-acids which disappeared from the circulation were absorbed, without suffering immediate chemical change, by the tissues.*

It will be noted that the amino nitrogen content of the muscles did not rise so high as that of the internal organs. The figures exemplify a fact that we have noted in all our experiments, viz., that the amount of amino-acid nitrogen that the muscles can hold is limited with relative sharpness. By injection of the amino-acids from proteins hydrolyzed by either acids or enzymes we have never been able to force the amino nitrogen figure of the striped muscle above 80 mg. per 100 grams. If the figure is above 70 at the time of injection, little or no amino nitrogen is taken up. In the liver we have noted as high as 160 mg., and the other internal organs seem to possess a more elastic ability to absorb amino-acids than do the muscles.

Experiment 2. The conditions were similar to those of the first experiment. The dog used for Experiment 2 weighed 7.4 kg, and the amount of amino nitrogen injected was 3.39 grams. In this case the second set of tissue samples was taken one hour after the injection had been finished.

TABLE II.

TISSUE SAMPLE	MG. AMINO NITROGEN PER 100 GRAMS TISSUE	
	Before injection	One hour after injection
Liver 1.....	48	
Liver 2.....	44	
Liver 3.....		127
Liver 4.....		124
Right gracilis 1.....	46	
Right gracilis 2.....	46	
Left gracilis 1.....		78
Left gracilis 2.....		76
Right kidney.....	52	
Left kidney.....		111
Blood 1.....	5.8	
Blood 2.....	5.9	
Blood 3.....		31.5

The amino nitrogen excreted in the urine during the diuresis following the injection was 0.552 gram, or 16.3 per cent of that injected.

Other experiments could be cited, but the above two appear to show with sufficient clearness the phenomena discussed in connection with the first experiment. The following serves as a control.

Experiment 3. A female bull terrier of 17 kg. weight, which had fasted twenty-four hours, was given during an hour an intravenous injection of 250 cc. of physiological saline solution. Samples of tissues and blood were taken before and after the injection as in the previous experiment.

TABLE III.
Injection of salt solution.

TISSUE SAMPLE	MG. AMINO NITROGEN PER 100 GRAMS TISSUE	
	Before injection	After injection
Right sartorius 1.....	59	
Right sartorius 2.....	67	
Left sartorius 1.....		61
Left sartorius 2.....		62
Liver 1.....	56	
Liver 2.....	54	
Liver 3.....		54
Liver 4.....		55
Liver 5.....		56
Kidney 1.....	43	
Kidney 2.....		43
Blood 1.....	4.9	
Blood 2.....		4.7

Diuresis followed the salt solution injection, 150 cc. of urine being excreted. The nitrogen excreted was 0.514 gram, of which, however, only 0.010 gram was amino-acid nitrogen. The concentration of the blood was controlled by determination of its total nitrogen content. It was practically the same (2.94 per cent) at the end of the experiment as at the beginning (2.92 per cent). The results in the above table show that the operation and injection of water solution cause no appreciable change in the amino nitrogen content of the blood or tissues.

Concerning the physiological effects of intravenous injection of amino-acid mixtures, we have noted from experiments, including others than those tabulated above, the following behavior. Mixed amino-acids, whether obtained by acid hydrolysis of casein or by artificial digestion of beef (with pepsin, trypsin, and erepsin, till 90 to 95 per cent of the maximum amount of NH_2 that can be freed by acid hydrolysis has been liberated), are tolerated by dogs in

doses up to 0.15–0.20 gram of amino nitrogen per kilo body weight, one hour or more being taken to complete the injection. During the latter no serious fall in blood pressure occurs, respiration appears normal and the animals apparently uninjured, as found by Buglia after similar injections. When the amino nitrogen injected exceeds the above limits, it may cause trembling, weakened heart action, fall in blood pressure, and death within an hour or two after the injection. Diuresis is abundant in these cases, and we have noted the excretion of as much as 500 cc. of urine, containing 20 per cent of the injected amino nitrogen. Doses under 0.15 gram of amino nitrogen per kilo usually cause but relatively moderate diuresis and excretion of amino-acids.

We have stated that amino-acids entering the blood stream are merely absorbed without chemical alteration by the tissues. This is a very loose description of a phenomenon for which the complete explanation will be far from easy. That the absorbed amino-acids enter into the organic structure of the tissue proteins, however, seems to be positively excluded by the fact that they can be removed again by such mild means as extraction with, not only hot water, but with cold water or alcohol. Of purely physical explanations, osmosis can be definitely excluded, because the ultimate concentration in the tissues is several times higher than in the blood. That the amino-acids should pass, so to speak, up hill, from a medium where they are dilute to one where they are more concentrated, requires another explanation than mere osmosis. It is possible that, having diffused into the cells, the amino-acids are fixed in a loose molecular combination by the proteins, as water of crystallization is held by salts, or as, according to Pfeiffer's recent results, the amino-acids themselves combine with neutral salts such as sodium and calcium chlorides.³⁷ A second possible explanation which is not yet ruled out by the facts is that of purely physical adsorption, the amino-acids being attracted to the colloids of the tissues by forces of surface tension or molecular attraction, such as enable charcoal or cloth fibers to adsorb dyes. We shall, however, leave the solution of this phase of the problem to the future, and merely use the term "absorption" to designate the phenomenon.

³⁷ Pfeiffer: *Zeitschr. f. physiol. Chem.*, lxxxi, p. 329, 1913.

SUMMARY.

The disappearance of intravenously injected amino-acids from the circulation is the result of neither their destruction, synthesis, nor chemical incorporation into the cell proteins. The acids are merely *absorbed from the blood by the tissues, without undergoing any immediate chemical change*. In the case of the muscles at least, a fairly definite saturation point exists, which sets the limit to the amount of amino-acids that can be absorbed. We have never been able to force the amino nitrogen figure of the striated muscles above 75–80 mg. per 100 grams. The capacity of the internal organs is more elastic; we have raised the amino figure of the liver to 125–150 mg.

The absorption of amino-acids from the circulation by the tissues, although extremely rapid, is never complete; the blood contains 3–8 mg. of amino-acid nitrogen per 100 cc. even after a fast of several days' duration. The amino-acids of the blood appear, therefore, to be *in equilibrium* with those of the tissues, a condition which accounts for all the observed phenomena, and would also account for any transfer of amino-acids which may occur from organ to organ, or from maternal organs to foetus.

The process by which the amino-acids are taken up and held by the tissues cannot be wholly osmotic, because the normal concentration of amino nitrogen in the tissues is five to ten times that in the blood; and even when the latter is suddenly loaded with injected amino-acids, they quickly gather in not equal, but greater, concentration in the tissues.³⁸ The most probable explanations of the process are, that it is either: (1) a mechanical adsorption, or (2) the formation of loose molecular compounds between the amino-acids and the tissue proteins, such as Pfeiffer has recently shown can be formed by the amino-acids themselves with inorganic salts. A discussion of this question would at present be premature.

³⁸ Further examples of this phenomenon are given in the experiments in the next paper.

THE FATE OF PROTEIN DIGESTION PRODUCTS IN THE BODY.

IV. THE LOCUS OF CHEMICAL TRANSFORMATION OF ABSORBED AMINO-ACIDS.¹

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After tracing the amino-acids from the intestine into the blood,² and from the blood into the tissues,³ the question next to offer itself concerns the duration of their stay in the various organs, and the nature of the changes which are responsible for their final disappearance. That the amino-acids do not long remain unaltered in the body follows from facts which are already known. Levene, working with Kober⁴ and Meyer,⁵ has found, for example, that alanine and arginine when fed to a dog are excreted almost completely in the form of urea in the next twenty-four hours. Also, when protein is added to the diet of an animal already in nitrogenous equilibrium, an increased excretion of urea follows, which during the next one or more days corresponds nearly to the amount of nitrogen in the added protein.

EXPERIMENTAL.

Technique. In order to obtain evidence on the point in question, experiments of the following nature were performed. Dogs were

¹ The results in this paper were reported in abstract at the meeting of the Society for experimental Biology and Medicine, Dec. 18, 1912. *Proceedings*, x, p. 38.

² This *Journal*, xii, p. 402, 1912.

³ *Ibid.*, preceding article.

⁴ *Amer. Journ. of Physiol.*, xxiii, p. 324.

⁵ *Ibid.*, xxv, p. 214.

injected intravenously with non-toxic doses of glycocoll, or of the mixtures of amino-acids obtained by hydrolysis of casein or complete artificial digestion of meat, these mixtures corresponding more nearly to those normally absorbed from the intestine than could a solution of a single amino-acid. After the injection, which consumed from an hour to an hour and a half, a half-hour was allowed to elapse for the absorption of the injected amino-acids by the tissues to become approximately complete. Then samples of the tissues (liver, muscle, kidney, intestine) were ligated or clamped off and removed for analysis. Several hours later similar samples were taken in order to ascertain which organs had retained their stored amino-acids and which had metabolized them, or begun to do so, most rapidly. During the experiments the blood and urine were also controlled by analysis. The animals were kept under ether by the efficient and convenient insufflation method of Meltzer and Auer.

The animals were for some days before the experiments either fasted or fed on a protein-free diet, in order to remove the products of protein digestion from the alimentary canal, and also, if possible, to decrease the concentration of amino-acids in the tissues. The former object was, of course, easily obtained, but the latter was not. As will be shown in the next paper, a regular decrease in the amino-acids of the tissues cannot be caused by removal of protein from the diet.

The solutions injected were allowed to flow at a regular rate from a burette into either the femoral or jugular vein, and were warmed by passing through a glass coil in a bath at 40° just before they entered the vein. The animals were warmed with an electric pad. Samples of arterial blood were taken before the injection, a half hour afterwards, and at the end of the experiment, two to four hours later. The samples, of about 25 cc. volume, were mixed with a little powdered sodium citrate and 1 to 2 cc. of the complete blood taken with a pipette for Kjeldahl determination, to control the concentration. The remainder of each sample was freed from protein by precipitation with 10 parts of 95 per cent ethyl alcohol, as described in our preliminary paper.⁶ The filtrate from the proteins was brought to 10 cc., of which 2-cc. portions were used for

⁶ *This Journal*, xii, p. 402, 1912.

determination of amino-acid nitrogen, 1 cc. for urea determination by the recent method of Folin.⁷

Before the injection samples of either the gracilis or triceps muscles were taken and analyzed for amino nitrogen by the method described in our second paper.⁸ The results serve to some extent to indicate the degree to which the tissues in general are saturated with amino-acids. The two samples after the injection, being used for comparison, were always the right and left gracilis or the right and left triceps. A gracilis cannot be accurately compared with a triceps (see following paper for examples) as the latter is usually somewhat higher in amino nitrogen. When kidneys were taken, either the entire organ was analyzed, or it was bisected longitudinally and the halves used for duplicates.

The amount of injected amino nitrogen, whether in the form of hydrolyzed casein, artificially digested meat, or pure amino-acids, was usually chosen at 0.15–0.20 gram per kilo body weight. Larger doses, although used in some experiments, are likely not to be tolerated by the animals (see Experiment 4, for example). Within a half-hour after the injection of the above amounts, the amino-acids have been almost completely absorbed from circulation by the tissues, the amino nitrogen content of the blood having fallen to 15–20 mg. per 100 cc. Samples of the tissues are then taken as described in a preceding paragraph.

Before the injections the animals were either catheterized and their bladders washed out, or cannulas were placed in the ureters. During the experiments the catheters were left in place in order to collect the urine that always begins to flow shortly after the injection is begun. The urine was collected in two periods, one extending from the beginning of the injection till the first tissue samples were taken after it; the other period being the remaining time of the experiment. The urines were analyzed for free amino-acid nitrogen,⁹ urea,¹⁰ ammonia, and total nitrogen, and the rotations were taken. For the latter, 2 cc. of concentrated hydrochloric acid were added to 10 cc. of urine; the mixture was cleared with

⁷ This *Journal*, xi, p. 507, 1912.

⁸ *Ibid.*, present number.

⁹ Levene and Van Slyke: *ibid.*, xii, p. 301, 1912; Van Slyke: *ibid.*, preceding number.

¹⁰ Folin: *ibid.*, xi, p. 507, 1912.

charcoal, and the rotation taken in a 2 dm. tube with yellow light from a spectroscope. In urines of dogs that had fasted before the injections the rotation usually corresponded to that of a solution of the injected amino-acids containing amino nitrogen in the same concentration as the urine. When the animals had received a protein-free diet of fat and starch, however, the urines were much more strongly dextrorotatory, and contained reducing sugar.

In part of the experiments, after the first blood sample had been drawn from the carotid artery the latter was connected with a mercury manometer, and the blood pressure during the injection and succeeding hours recorded. Amino-acid mixtures containing sufficient nitrogen to keep the animals in equilibrium for twenty-four hours could usually be injected without causing a drop in blood pressure. Opening the abdomen and manipulating the viscera to remove samples caused a quick drop to, as a rule, about 60 mm. Subsequently the original blood pressure was partly, sometimes almost entirely, reestablished.

All duplicate analyses reported were made on separate portions of blood or tissue.

Experiment 1. The animal, a rather lean bitch in good condition, was fasted four days before the experiment, its weight falling from 11 kilos to 10.3. The animal was etherized, samples of the right gracilis muscle removed, and blood samples drawn from the right femoral artery. A catheter was inserted and the bladder washed out. A solution of hydrolyzed casein¹¹ containing 2.70

¹¹ Casein was hydrolyzed by forty-eight hours' boiling with 7 parts of 33 per cent sulphuric acid. The latter was removed with an excess of barium hydrate, and the ammonia driven off by concentration in vacuum. The barium was removed with sulphuric acid, just enough of the latter being used so that a slight test for sulphate could be detected in the solution. The latter was concentrated in vacuum, and made just perceptibly alkaline to litmus with sodium carbonate. The rotation of the solution was determined on the basis of the amino nitrogen content, in order that the results might be used for comparison with those of urine analyses. 1.000 cc. of the solution was mixed with 2 cc. of concentrated HCl, diluted to 10 cc., and cleared with charcoal. In a 2 dm. tube the rotation was $+0.50^\circ$. The amino nitrogen content of the tenfold diluted solution was 0.00271 gram per cc. The rotation, calculated by the formula,

$$\alpha_{\text{NH}_2} = \frac{\text{observed rotation of 1 dm. layer of solution}}{\text{grams amino N per cc.}}, \text{ is } \frac{0.25^\circ}{0.00271} = +92^\circ.$$

grams of amino nitrogen in 250 cc. was injected into the femoral vein during one hour and ten minutes. Shortly after the injection was begun urine began to drop from the catheter; the amount passed during the injection and the succeeding half hour was 260 cc.; during the next two hours only 25 cc. additional were excreted.

An hour after the injection had been finished samples of the right triceps muscle and a lobe of the liver were removed. Two and a half hours later the animal was bled to death, samples of the left triceps and of other lobes of the liver being taken. The results are given in Table I.

The blood pressure of this animal was not followed, but it seemed to tolerate without difficulty the unusually large dose of amino-acids, 0.26 gram of amino nitrogen per kilo. The analyses were made by the "absolute method" described in the second paper of this series.

TABLE I.

Weight of dog, 10.3 kilos. Amino nitrogen injected, 2.70 grams. Amino nitrogen excreted, 0.55 gram.

TISSUE SAMPLE	TIME OF EXCISION	AMINO NITROGEN PER 100 GRAMS TISSUE
		mgm.
Muscle. Right gracilis 1.....	Before injection.	45
Muscle. Right gracilis 2.....	Before injection.	47
Muscle. Right triceps 1.....	1 hour after injection.	68
Muscle. Right triceps 2.....	1 hour after injection.	63
Muscle. Left triceps 1.....	3 hours after injection.	67
Muscle. Left triceps 2.....	3 hours after injection.	65
Liver 1.....	1 hour after injection.	79
Liver 2.....	1 hour after injection.	85
Liver 3.....	3 hours after injection.	34
Liver 4.....	3 hours after injection.	39

The peptide-bound amino nitrogen in all the tissue samples was also determined, but it showed no significant changes.

The urine in this case shows, unlike that of most fasted animals, a much higher rotation than would be calculated from the content of amino-acids. The reducing power of this urine was not determined.

At the end of the experiment 5 cc. of bile were expressed from the gall bladder. Neither the bladder nor the intestine indicated that

an unusual excretion of bile had occurred. The amino nitrogen content, determined without removal of the mucin, was only 0.12 mg. per cubic centimeter. The amino-acids which disappeared from the liver could not, to a significant extent, have been excreted in the bile.

TABLE II.
Blood analyses. Experiment 1.

NO.	TIME	AMINO N PER 100 CC.	UREA N PER 100 CC.
		mgm.	mgm.
1	Before injection.....	8.2	11
2	Before injection.....	7.8	12
3	1 hour after injection.....	18.3	22
4	1 hour after injection.....	18.6	21
5	3 hours after injection.....	12.4	23
6	3 hours after injection.....	11.9	23

TABLE III.
Urine analyses. Experiment 1.

	URINE FROM BEGIN- NING OF INJECTION TILL 1 HOUR AFTER INJECTION	URINE DURING NEXT 2 HOURS
Volume.....	260 cc.	25 cc.
Total nitrogen.....	0.854 gram	0.045 gram
Amino nitrogen.....	0.530 gram	0.018 gram
Observed rotation in 2 dm. tube of urine diluted with 0.25 volume con- centrated HCl.....	+0.50°	
* α_{NH_3}	+153.00°	

* See footnote 11, page 216.

We interpret the results as follows. As the result of the injection the amino figure of the muscles rose to 66 and remained near the latter point for the following three hours, no noticeable amounts of the absorbed amino-acids being destroyed or synthesized into protein in the muscles, or removed from them during this period. In the liver the case is altogether different. As the result of absorption of injected amino-acids the concentration in the liver rose, as usual (see preceding paper), even higher than in the muscles. It fell again with almost startling rapidity, however, dropping between the second and fourth hours after the injection from 79-85

mg. to 34-39 mg. per 100 grams of fresh tissue. The latter figure is undoubtedly about as low as before the injection, for we have never found the amino figure in the livers of normal dogs, fasted or fed, below 30 mg. Presumably the fall had already begun before the first samples were taken, one hour after the end of the injection, two hours after it had been begun. For the nature of the change involving the disappearance of injected amino-acids from the liver the following explanations might be proposed.

1. The amino-acids were excreted. This explanation is entirely inadequate. The bile contained very little amino nitrogen, including that of its protein; and in the urine only 18 mg. of amino nitrogen were excreted during a period when 250-300 mg. disappeared from the liver.

2. The amino-acids were transferred to other tissues. This seems most improbable. None of the other large organs shows a greater avidity for amino-acids, to judge from the amounts absorbed (see paper preceding this), yet three or four hours after the injection all usually contain much more than the liver (compare following experiments). That the absorbed amino-acids should have been removed from the liver and concentrated in the other organs, after all had in free competition taken up their shares from the blood, is improbable.

3. The absorbed amino-acids are synthesized into body protein in the liver. Concerning this possibility we have at present no evidence on which we can decide in one way or another. It is possible that at least a part of the amino-acid mixture is immediately resynthesized into protein. There is, however, at present no positive evidence that this is the case. If a rapid synthesis were occurring in the liver one might expect to find an increase in the intermediate products indicated by the peptide bound nitrogen in the extract. We found no such increase, this nitrogen being about 20 mg. one hour, and the same three hours after the injection. Furthermore, the fall in the amino nitrogen of the liver following the injection of glycocoll is similar to that observed after injection of hydrolyzed protein (see Experiment 5). That glycocoll by itself should be turned into body protein appears impossible.

4. The amino-acids are deaminized with formation of urea or ammonia. If ammonia is formed it presumably undergoes further

transformation at once; for during the disappearance of amino-acids from the liver we have been able to find no increase in the ammonia content of that organ (see next experiment), nor is the ammonia excretion in the urine marked. That at least part of the amino-acid nitrogen is transformed into urea is, however, definitely indicated by the increase in urea nitrogen of the blood following the injection of amino-acids.

The volume of the blood in the dog is, at a rough approximation, equal to that of the liver. In case the amino-acid nitrogen which disappears from the latter were transferred entirely and exclusively to the blood in the form of urea, we should expect the urea concentration of the blood to show a corresponding increase (only a small amount of urea escapes by way of the urine during the last period of the experiment). As a matter of fact, only a fraction of the amino nitrogen which disappears from the liver reappears as urea in the blood. It is possible, however, that the urea in the blood is, like the amino-acids, in equilibrium with that of the tissues, in which case urea entering the blood from the liver would, unless immediately excreted, be partially taken up by the other tissues. In a number of experiments we have analyzed the tissues for urea in order to test this point, but are not sufficiently satisfied with the reliability of the methods to report the results at present.

Experiment 2. The conditions were similar to those in Experiment 1, the chief difference being that the dose of amino-acids was smaller, so that the results were somewhat less pronounced. The animal, having fasted six days, weighed 12.8 kilos. He was etherized, and, as before, samples of blood and muscle were taken. 125 cc. of hydrolyzed casein, containing 1.90 grams of amino nitrogen (0.149 gram per kilo) were injected into the right femoral vein, one hour being taken for the injection. Samples of blood and tissues were taken thirty minutes after the injection was finished, and again three hours later.

The total amino nitrogen excreted was 0.12 gram, 6 per cent of the amount injected. The rotation of the chief fraction of the urine calculated on the basis of the amino nitrogen agrees closely with that of the injected solution, indicating that the different amino-acids were probably excreted in nearly the same proportions in which they were injected.

The results of this experiment demonstrate the same facts as the one preceding. In addition, the analyses of samples of kidney,

TABLE IV.

Tissue analyses. Experiment 2.

Weight of dog, 12.8 kilos. Amino nitrogen injected (hydrolyzed casein), 1.90 grams. Amino nitrogen excreted, 0.12 gram.

TISSUE SAMPLE	TIME OF EXCISION	AMINO NITROGEN PER 100 GRAMS	AMMONIA NITROGEN PER 100 GRAMS
		mgm.	mgm.
Right gracilis 1.....	Before injection.	49	12
Right gracilis 2.....	Before injection.	49	13
Right triceps 1.....	0.5 hour after injection.	65	13
Right triceps 2.....	0.5 hour after injection.	68	13
Left triceps 1.....	3.5 hours after injection.	66	12
Left triceps 2.....	3.5 hours after injection.	67	14
Liver 1.....	0.5 hour after injection.	58	10
Liver 2.....	0.5 hour after injection.	57	11
Liver 3.....	3.5 hours after injection.	33	12
Liver 4.....	3.5 hours after injection.	33	10
Pancreas.....	3.5 hours after injection.	63	9
Kidney.....	3.5 hours after injection.	50	10
Duodenum.....	3.5 hours after injection.	64	12

TABLE V.

Blood analyses. Experiment 2.

NO.	TIME TAKEN	AMINO N PER 100 CC.	UREA N PER 100 CC.	TOTAL N PER 100 CC.
		mgm.	mgm.	grams
1	Before injection.....	4.2	8	3.25
2	Before injection.....	3.9	8	
3	0.5 hour after injection.....	19.8	11	2.91
4	0.5 hour after injection.....	19.6		
5	3.5 hours after injection.....	7.5	14	3.14
6	3.5 hours after injection.....	8.2	13	

TABLE VI.

Urine analyses. Experiment 2.

	URINE FROM BEGIN- NING OF INJECTION TILL ONE-HALF HOUR AFTER INJECTION	URINE DURING NEXT THREE HOURS
Volume.....	43 cc.	12 cc.
Total nitrogen.....	0.213	
Amino nitrogen.....	0.100	0.021
Urea + ammonia nitrogen.....	0.058	0.018
Observed rotation in 2 dm. tube of urine diluted with 0.2 volume con- centrated HCl.....	+0.41°	+0.59°
* α_{NH_3}	+88°	+111°

* See footnote 11, page 216.

pancreas, and intestinal wall taken three and one-half hours after the injection indicate that deaminization in these tissues is not so rapid as in the liver, for the amino nitrogen content in none of them has sunk so low as in the liver.

Experiment 3. This experiment was similar to the preceding except that instead of the mixture of amino-acids obtained by acid hydrolysis of casein, that obtained by digestion of meat with pepsin, trypsin, and erepsin was injected.¹² The animal was kept for eight days before the experiment on a protein-free diet of starch, lard, and salts. The animal weighed 13.3 kilos before the protein-free diet was begun, 11.6 kilos at the time of the experiment. The bladder was washed out through a catheter as usual. The blood samples were drawn from the carotid artery, and the injection made into the jugular vein. The 185 cc. of solution injected contained 2.06 grams of amino nitrogen, a dose of 0.18 gram per kilo.

The muscles of this animal were, to judge from their high amino nitrogen content before the injection, filled with amino-acids before the experiment to a degree unusually close to saturation (see summary of preceding paper). This is probably the reason why the comparatively moderate injection of amino-acids forced the amino content of the liver to such an unusual height. As the result of this height, the succeeding drop is exceptionally great.

There was apparently no change in the amino-acid content of the kidneys in the period between thirty minutes and four hours after the injection. There was also little excretion of urine (7 cc.) during this period, although 240 cc. had been excreted during the injection and the first half hour thereafter.

From comparison of the triceps muscle before injection with the gracilis after (Table VII), one might judge that the muscle had taken up none of the injected amino-acids, as the figures are about equal before and after. Reference to the table in the next paper

¹² Beef was ground in a machine and boiled. It was then digested a week with pepsin, two weeks with trypsin, which was added in fresh portions every few days, and a month with the mucous membrane of dogs' intestines. Toluene and chloroform together were used to insure antisepsis. The course of the digestion was followed by amino determinations. At the time it was stopped it was 90 per cent complete, taking the amino nitrogen freed by boiling twenty-four hours with 20 per cent HCl as indicating 100 per cent. The antiseptics were removed by concentration in vacuum, and the solution boiled, then preserved at 0°.

TABLE VII.

Tissue analyses. Experiment 3.

Weight of dog (eight days on protein-free diet), 11.6 kilos. Amino nitrogen (digested beef) injected, 2.06 grams. Amino nitrogen excreted, 0.318 gram.

TISSUE SAMPLE	TIME OF EXCISION	AMINO N PER 100 GRAMS
Muscle. Triceps 1.....	Before injection.	70
Muscle. Triceps 2.....	Before injection.	65
Muscle. Right gracilis 1...	0.5 hour after injection.	67
Muscle. Right gracilis 2...	0.5 hour after injection.	73
Muscle. Left gracilis 1....	4 hours after injection.	70
Muscle. Left gracilis 2....	4 hours after injection.	73
Liver 1.....	0.5 hour after injection.	156
Liver 2.....	0.5 hour after injection.	157
Liver 3.....	4 hours after injection.	69
Liver 4.....	4 hours after injection.	73
Right kidney.....	0.5 hour after injection.	88
Right kidney.....	4 hours after injection.	89

TABLE VIII.

Blood analyses. Experiment 3.

NO.	TIME	AMINO N PER 100 CC.	UREA N PER 100 CC.	TOTAL N PER 100 CC.
		mgm.	mgm.	grams
1	Before injection.....	4.7	5	3.33
2	0.5 hour after injection.....	13.7	10	3.28
3	4 hours after injection.....	11.0	14	3.21

TABLE IX.

Urine analyses. Experiment 3.

	URINE FROM BEGINNING OF INJECTION TILL 1 HOUR AFTER INJECTION	URINE DURING NEXT THREE AND ONE-HALF HOURS
Volume.....	240 cc.	7 cc.
Total nitrogen.....	0.826 gram	0.025 gram
Amino nitrogen.....	0.318 gram	
Urea nitrogen.....	0.107 gram	
Ammonia nitrogen.....	0.024 gram	

shows, however, that the amino-acid nitrogen in the triceps muscles is normally 10-20 mg. higher than in the gracilis. The figures in Table VII indicate, therefore, that the amino nitrogen of the gracilis was probably raised at least 10 mg. per 100 gm. by the injection.

Experiment 4. This experiment shows the typical results of an overdose of injected amino-acids. The animal, a female in the early stage of pregnancy, was on a protein-free diet for nine days before the experiment. 250 cc. of a solution of hydrolyzed casein, containing 3.81 grams of amino nitrogen, were injected. The dose,

TABLE X.

Tissue analyses. Experiment 4.

Weight of dog (nine days on protein-free diet), 17.4 kilos. Amino nitrogen (hydrolyzed casein) injected, 3.81 grams. Amino nitrogen excreted, 0.873 gram.

TISSUE SAMPLE	TIME OF EXCISION	AMINO NITROGEN PER 100 GRAMS
Muscle. Right gracilis	Before injection.	36
Muscle. Right triceps	0.5 hour after injection.	56
Muscle. Left triceps	2½ hours after injection.	55
Liver 1	0.5 hour after injection.	53
Liver 2	0.5 hour after injection.	58
Liver 3	2½ hours after injection.	45
Liver 4	2½ hours after injection.	48
Right kidney 1	0.5 hour after injection.	60
Right kidney 2	0.5 hour after injection.	69
Left kidney 1	2½ hours after injection.	48
Left kidney 2	2½ hours after injection.	43
Duodenum	2½ hours after injection.	53
Spleen	2½ hours after injection.	76
Pancreas	2½ hours after injection.	66

0.21 gram of amino nitrogen per kilo, was but slightly above the usual tolerated amount. This animal, however, reacted at once with a profuse diuresis, 450 cc. of urine being excreted during the ninety minutes of the injection and the following thirty minutes. 200 cc. of normal saline solution were injected to replace the volume of water lost by the animal. The diuresis continued, 155 cc. more of urine being voided during the succeeding two hours. The heart weakened before the injection was finished and the breathing became shallow and irregular. Two hours after the finish of the

TABLE XI.
Blood analyses. Experiment 4.

NO.	TIME	AMINO N PER	UREA N PER	TOTAL N PER
		100 cc.	100 cc.	100 cc.
		mgm.	mgm.	grams
1	Before injection	10	10	3.32
2	Before injection	9	11	
3	0.5 hour after injection	31	16	3.03
4	0.5 hour after injection	30	16	
5	2 $\frac{1}{4}$ hours after injection	20	14	3.40
6	2 $\frac{1}{4}$ hours after injection	20	14	

TABLE XII.
Urine analyses. Experiment 4.

	URINE FROM BEGIN- NING OF INJECTION TILL 0.5 HOUR AFTER INJECTION	URINE FROM 0.5 HOUR AFTER INJECTION TILL EXITUS
Volume.....	450 cc.	155 cc.
Total nitrogen.....	1.163 grams	0.362 gram
Amino nitrogen.....	0.658 gram	0.215 gram
Urea nitrogen.....	0.191 gram	0.033 gram
Ammonia nitrogen.....	0.070 gram	0.019 gram
Observed rotation (2 dm. tube) of urine diluted with 0.2 volume con- centrated HCl.....	+1.02°	+0.92°
Glucose (reduction).....	0.87 per cent	0.77 per cent
Rotation due to amino-acids, calcu- lated from $\alpha_{NH_2} = +92^\circ$	+0.26°	+0.24°
Rotation not due to amino-acids....	+0.76	+0.68
Glucose calculated from rotation not due to amino-acids.....	0.87 per cent	0.79 per cent

* See footnote 11, page 216.

injection a convulsion occurred, and forty-five minutes later the heart stopped.

The animal exhibited an abnormal behavior in a number of ways. Despite the unusual proportion (23 per cent) of the injected amino nitrogen excreted in the urine, the amino content of the blood did not return so rapidly nor so nearly to normal as in the preceding experiments. The urea of the blood, instead of increasing somewhat during the last period, fell 2 mg.

As usual with dogs fed on fat and carbohydrate before the in-

jection, this was followed by a marked glucosuria, shown by both rotation and reducing power of the urine. Urine excreted shortly before the injection contained no sugar.

The nitrogen of the urine belonged chiefly to the amino-acids. In the hydrolyzed casein, freed of ammonia, which was injected, only 80 per cent of the total nitrogen is in the form of NH_2 , the other 20 per cent being due to the proline, arginine and other amino-acids containing non-amino nitrogen. Therefore, the free amino nitrogen must be increased by one-fourth in order to calculate approximately the actual amount of amino-acid nitrogen present. The 0.658 gram of NH_2 nitrogen excreted in the first period indicates that actually 0.82 of the 1.16 grams of nitrogen excreted was in amino-acids.

Both absorption of amino-acids by the liver (to judge from its analysis thirty minutes after the injection) and the subsequent decrease were small. In the kidneys, on the other hand, a marked fall occurred in the amino nitrogen content during the last period ($\frac{1}{2}$ to $2\frac{3}{4}$ hours after the injection). The most probable explanation of this seems to be that the amino-acids were washed out of the remaining kidney (one having been removed thirty minutes after the injection) by the active diuresis. A similar fall has been observed in two other experiments (unpublished), in each of which the diuresis continued into the last period of the experiment. Usually diuresis ceases shortly after the injection, and the amino content of the kidney does not fall markedly during the last period. We have other experiments under way to determine whether the explanation suggested is correct or not.

Experiment 5. This experiment was similar to the others, except that glycocoll instead of the mixture of amino-acids obtained by hydrolysis of a protein, was injected. The results were similar to those obtained with moderate doses of the mixtures. A rapid disappearance of absorbed amino-acids occurred from the liver, but not from the muscles or kidney. The spleen and pancreas were found loaded with amino nitrogen at the end of the experiment.

The injected solution contained 9.3 grams of glycocoll in 125 cc. of water, the dose of amino nitrogen per kilo (weight of dog, 10.9 kg.) being 0.16 gram. The injection was made into the jugular vein, one and one-half hours being taken to complete it. The

blood pressure remained at 145–150 mm. during the injection. When the liver samples were taken after the injection the pressure dropped to 60 mm. It rose again to 105 mm. during the next hour and a half, and remained at 95–105 during the rest of the experiment.

TABLE XIII.

Tissue analyses. Experiment 5.

Weight of dog (two days fast), 10.9 kilos. Amino nitrogen injected (glycocoll), 1.74 grams. Amino nitrogen excreted, 0.06 gram.

TISSUE SAMPLE	TIME OF EXCISION	AMINO NITROGEN PER 100 GRAMS
Right gracilis muscle 1	30 minutes after injection.	62
Right gracilis muscle 2	30 minutes after injection.	68
Left gracilis muscle 1	3 hours after injection.	67
Left gracilis muscle 2	3 hours after injection.	61
Liver 1	30 minutes after injection.	61
Liver 2	30 minutes after injection.	57
Liver 3	3 hours after injection.	43
Liver 4	3 hours after injection.	43
Kidney 1	30 minutes after injection.	68
Kidney 2	3 hours after injection.	65
Duodenum	3 hours after injection.	66
Spleen	3 hours after injection.	129
Pancreas	3 hours after injection.	169

TABLE XIV.

Blood analyses. Experiment 5.

TIME	AMINO N PER 100 CC.	UREA N PER 100 CC.	TOTAL N PER 100 CC.
	<i>mgm.</i>	<i>mgm.</i>	<i>grams</i>
Before injection	5.6	14	3.99
30 minutes after injection	22.5	16	3.80
3 hours after injection	13.4	27	3.89

The urine excreted measured only 25 cc. in all, and contained 0.203 gram nitrogen, of which but 0.061 gram was amino-acid nitrogen, this amount being 3.5 per cent of that injected. The tendency to excrete injected amino nitrogen appears to be somewhat less when the single amino-acid, glycocoll, is injected than when an equal dose in the form of the mixture of amino-acids obtained by hydrolysis of a protein enters the circulation.

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Experiment 6. Control, saline injection. This experiment was performed in order to ascertain whether the operative treatment and injection of liquid could have produced any changes in the amino nitrogen of the liver or kidney. A dog of 11 kg. received in one hour 200 cc. of normal saline, which was injected into the jugular vein. Thirty minutes after the injection was finished a lobe of the liver and one of the kidneys were removed for analysis. Three hours later similar samples were taken. The results in the table below show no changes in the amino figures during this period.

TABLE XV.

Tissue analyses. Experiment 6.

Weight of dog (fasted twenty-four hours), 11 kilos. Injection of 200 cc. of 0.8 per cent NaCl solution, no amino nitrogen.

TISSUE SAMPLE	TIME OF EXCISION	AMINO NITROGEN PER 100 GRAMS
Liver 1.....	0.5 hours after injection.	42
Liver 2.....	0.5 hour after injection.	47
Liver 3.....	3.5 hours after injection.	45
Liver 4.....	3.5 hours after injection.	41
Liver 5.....	3.5 hours after injection.	42
Right kidney.....	0.5 hour after injection.	48
Left kidney.....	3.5 hours after injection.	48

The amino acid nitrogen of the blood remained unchanged throughout the experiment at 5 mg. per 100 cc., and the urea nitrogen at 8 mg.

CONCLUSION.

In the preceding paper we have shown that amino-acids injected into the circulation are absorbed by the tissues. In the present paper it is shown that the absorbed amino-acids (glycocoll, hydrolyzed casein, artificially digested flesh) disappear rapidly from the liver. The amino nitrogen content of this organ may be doubled by an injection of amino-acids into the general circulation, and yet return to normal within two or three hours. During the period required by the liver to entirely rid itself of absorbed amino-acids, their concentration in the muscles suffers no appreciable fall. From the other organs (kidney, intestine, pancreas, spleen) the absorbed

amino-acids disappear less rapidly than from the liver, but whether as slowly as from the muscles has not yet been determined. The disappearance of amino-acids from the liver is accompanied by an increase in the urea of the blood. The results have been discussed in more detail on pp. 219 and 220.

These results support the long contended view¹³ that the liver is the organ especially responsible for the catabolism of those protein digestion products not utilized for tissue construction. The following explanation is consistent with the facts thus far ascertained. The amino-acids, with perhaps some peptides, from the intestine enter the circulation, from which they are almost immediately absorbed by the tissues. The power to take them up from the blood stream is common to all the tissues, but is limited. The muscles of the dog, for example, reach the saturation point when they contain about 75 mgm. of amino acid nitrogen per 100 grams. The liver, however, continually desaturates itself by metabolizing the amino-acids that it has absorbed, and consequently maintains indefinitely its power to continue removing them from the circulation, so long as they do not enter it faster than the liver can metabolize them. When the entrance is unnaturally rapid, as in our injection experiments, or when the liver is sufficiently degenerated, as observed clinically in some pathological conditions, the kidney assists in removing the amino-acids by excreting them unchanged. Death may result when the above agencies for preventing undue accumulation of protein digestion products are overtaxed (see Experiment 5).

In regard to the synthesis of tissue proteins, it appears reasonable to believe that, since each tissue has its own store of amino-acids, which it can replenish from the blood, it uses these to synthesize its own proteins.

¹³ Münzer and Winterberg: *Arch. f. exp. Path. u. Pharm.*, xxxiii, p. 163.

THE FATE OF PROTEIN DIGESTION PRODUCTS IN THE BODY.

V. THE EFFECTS OF FEEDING AND FASTING ON THE AMINO-ACID CONTENT OF THE TISSUES.

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In regard to the manner in which the free amino-acids stored in the tissues are utilized we may assume two possibilities.

1. The amino-acids serve as a reserve energy supply, like glycogen, or as a reserve of tissue building material. In either case the supply would be depleted if not renewed from the food.

2. The amino-acids are merely intermediate steps in both the construction and breakdown of the tissue proteins. In this case they could originate, not only from absorbed food products, but also from autolyzed tissue protein: starvation would not result in a disappearance of the amino-acid supply of the tissues, and might even increase it.

In order to obtain evidence on the point, the tissues of dogs in various states of nutrition were analyzed for free amino-acid nitrogen by the methods described in our second paper. All the analyses were conducted in the same manner, the ammonia being removed from the extracts before the amino nitrogen was determined. The dogs were killed by bleeding. Only males were utilized, in order that possible complicating effects of pregnancy might be avoided. The results, given in the accompanying table, indicate milligrams of amino nitrogen found per 100 grams of fresh tissue. They are decisively in accord with the second of the two above explanations. The free amino-acids of the tissues do not disappear during fasting; if anything, they tend to increase.¹

¹ Buglia and Constantino report also an increase in the "formoltitrier-bar" nitrogen (ammonia+amino-acids+amines) of the muscles during fast-

No.	1	2	3	4	5	6	7
PREVIOUS TREATMENT	MALE. REGULAR MIXED DIET TILL DAY OF DEATH. THEN RECEIVED 1 LB. FRESH BEEF FIVE HOURS BEFORE KILLED	MALE. WT. 16 KG. FED 500 GRAMS BEEF DAILY IN ADDITION TO REGULAR DIET FOR SEVEN DAYS BEFORE DEATH. LAST MEAL TWENTY HOURS BEFORE KILLED	MALE. WT. 16 KG. NORMAL DIET. LAST MEAL EIGHTEEN HOURS BEFORE DEATH. CHYME STILL IN INTESTINE	MALE. WT. 12 KG. FASTED FORTY- EIGHT HOURS	MALE. WT. 10 KG. FASTED FOUR DAYS	MALE. WT. 12 KG. FASTED SIX DAYS	MALE. FASTED TWELVE DAYS. WT. BEFORE FAST, 16 KG., AFTER FAST, 12.8 KG.
Right gracilis muscle.....	66	51	57	53	46	64	60
Left gracilis muscle.....	67	52	56	54	52	58	61
Right triceps muscle.....		61	80	64	61	71	
Left triceps muscle.....		58	78	64	64	72	
Liver, lobe 1....	43	59	85	69	70	93	95
Liver, lobe 2....	44	55	86	64	68	87	85
Liver, lobe 3....		64	78	73	71	85	90
Right kidney....	40	56	64	45	84	70	85
Left kidney.....		50	79	48	85	71	97
Pancreas.....	66	61	107	55	80	74	79
Spleen.....	70	92	147	69	93	99	
Duodenum.....		72	127	69	75	82	73
Jejunum.....	76	54	101	45	49	67	71
Ileum.....		54	70	36	43	74	43
Blood.....	8	6	8	6	7	5	5

The amino-acids appear, therefore, to be intermediate steps, not only in the synthesis, but in the breaking down of body proteins. Otherwise, in order to explain their maintenance in the tissues during starvation, one would be forced, contrary to the conclusions of all experimental work on the subject,² to assume that they are inert substances, lying unchanged for long periods, even

ing. They find in normal dogs 77-84 mg. "formol" nitrogen per 100 grams fresh muscle. In a dog that had fasted twelve days the figure was 95; sixteen days, 91; twenty days, 105; twenty-five days, 100.

² See articles by Levene, Kober, and Meyer cited in fourth paper.

when most urgently needed to build tissue or supply energy. The maintenance of the amino-acid supply by synthesis, from ammonia and the products of fats or carbohydrates, seems excluded. The supply of raw material in the form of fat and carbohydrates nearly disappears during starvation, and the ammonia could originate only from broken-down protein, as the normal store of ammonia nitrogen is only a fraction of that of the free amino-acids. These considerations, and the self-evident wasting of starved tissues, point strongly to autolysis as the main source of the free amino-acids in the fasting body.

The failure to increase the free amino-acid content of the tissues by high protein feeding indicates, furthermore, that when nitrogen is retained in the organism it is not, to an appreciable extent, as stored digestion products, but rather as body protein.



THE INFLUENCE OF SALTS COMMON IN ALKALI SOILS UPON THE GROWTH OF THE RICE PLANT.

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(Received for publication, August 29, 1913.)

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PART I. INFLUENCE OF SINGLE SALTS UPON THE GROWTH OF RICE SEEDLINGS.

Investigations on the influence of the salts common in alkali soils upon the growth of young seedlings have been made by many authors. In 1887, Hindolf¹ observed a good influence of magnesium and calcium chloride upon the early development of many cultivated plants. Coupin² studied the toxic influence of many salts upon the growth of the young root of wheat and found that calcium chloride was toxic in concentration of $\frac{N}{200}$. Hébert³ also investigated the toxicity of chromium, aluminium and magnesium salts upon the growth of germinated seeds of wheat and rape and observed that the toxic action of magnesium

¹ *Jost. bot. Jahrb.*, i, p. 139, 1887.

² *Compt. rend. de l'Acad. des Sci.*, cxxxii, p. 645, 1901.

³ *Bull. soc. chim. de France* (iv), i, p. 10-26, 1907; *Dietrichs' Jahrb.* *Agrik.-Chem.*, xi, p. 252, 1908.

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salts was least among these salts and often harmless. Since the discovery of large areas of alkali soils in western parts of the United States, the toxicity of the various salts common in alkali soils upon the growth of plants has been studied by many American authors, especially by Kearney and Harter.⁴ Their results appear in the following table.

Critical concentrations of pure solutions.

SALTS USED	PLANTS TESTED								
	WHITE LUPINE		ALFALFA	WHEAT	MAIZE	SORGHUM	OATS	COTTON	BEET
	I	II							
MgSO ₄	0.00125 N	0.007 N	±0.001 N	0.005 N	0.25 N	0.001875 N	0.001875 N	0.000312 N	0.0005 N
MgCl ₂	0.0025 N	0.0075 N	±0.002 N	0.005 N	0.08 N	0.001875 N	0.001875 N	0.0004 N	0.0005 N
Na ₂ CO ₃	0.005 N	0.0125 N		0.0125 N	0.015 N	0.00625 N	0.00625 N	0.005 N	0.00925 N
Na ₂ SO ₄	0.0075 N	0.04 N		0.04 N	0.05 N	0.0175 N	0.0175 N	0.005 N	0.00875 N
NaCl.....	0.02 N	0.045 N		0.045 N	0.04 N	0.02 N	0.02 N	0.00625 N	0.025 N
NaHCO ₃	0.02 N	0.03 N		0.025 N	0.05 N	0.0075 N	0.0075 N	0.00625 N	0.0075 N

They concluded that different species differ vastly in the absolute degree of their resistance to the toxic action of these pure solutions, also the order of toxicity of the several salts varies considerably according to the species. Furthermore, the salts of magnesium are generally more toxic than those of sodium to all the plants tested with the single exception of maize.

Burlingham⁵ has studied the influence of magnesium sulphate upon the growth of seedlings of abutilon, pea and corn, and his results were summarized as follows:

Magnesium sulphate in solutions of greater concentrations than $\frac{M}{8192}$ has a toxic action on most seedlings, the degree of toxicity varying with the type of seedlings and with the conditions. An $\frac{M}{8192}$ solution is toxic to pea seedlings, slightly stimulating to abutilon, while it has a marked stimulating effect on corn seedlings. Maximum stimulation in magnesium sulphate results in solution from $\frac{M}{32768}$ to $\frac{M}{131072}$, the point again varying according to the kind of seedling grown. When magnesium sulphate is used in proper dilutions there may be produced a total growth nearly double that in the control: or in the case of abutilon seedlings, a growth of the primary root increased, but the lateral roots develop sooner, are more

⁴Bulletin No. 13, Bureau of Plant Industry, U. S. Dept. of Agriculture, 1907.

⁵Journ. Amer. Chem. Soc., xxix, pp. 1095-1112, 1907.

numerous, and attain a greater growth. Furthermore the stimulation is not limited to the root system, but the magnesium forces a more rapid and a greater growth of the hypocotyl and plumule. In this same concentration, calcium nitrate causes very little stimulation.

In addition to the marked stimulation which magnesium sulphate causes when it is used in dilutions from $\frac{M}{10000}$ to $\frac{M}{521288}$, it increases the vitality of the seedlings. The seedlings grown in the magnesium sulphate outlived those in the control by two or three weeks, and in some cases by a greater period.

From the foregoing results and conclusions, it is then evident that magnesium sulphate, in the absence of other salts, is not necessarily injurious in its effects, but on the other hand may be highly beneficial; while any inhibitory action is due to the presence of a relatively large proportion of magnesium in the solution.

From the preceding investigations, it will be observed that the salts act on the growth of young seedlings as toxic or stimulating agents according to their concentrations.

In regard to the influence of these salts upon the growth of the rice plant, which is the most important crop in our country, a special investigation has not been made to date. But in 1909 the widely distributed alkali soils were discovered by Prof. Dr. K. Oshima and K. Shibuya, the Chemist of the Formosa Government, in the southern part of Formosa, and now it has become a most important subject of study. We undertook this study at the suggestion of Prof. Dr. K. Oshima, in order to find out the influence of the alkali salts upon the growth of rice seedlings, and selected magnesium sulphate, magnesium chloride, calcium chloride, sodium sulphate, sodium chloride, sodium carbonate and bicarbonate as the salts to be examined.

Experiment I.

In the first experiment we began with the young rice seedlings, 15-16 mm. high, which were grown in distilled water from seeds which were almost uniform in size and specific gravity (1.2-1.25). Fifty-six beakers of about 5.5 cm. diameter and 7 cm. deep, each containing 50 cc. of $\frac{M}{2}$, $\frac{M}{10}$, $\frac{M}{20}$, $\frac{M}{100}$, $\frac{M}{200}$, $\frac{M}{1000}$, $\frac{M}{2000}$, $\frac{M}{10000}$ solution of each salt mentioned above, were used for the experiment, the seedlings being placed in the solutions on August 3, 1911. A control experiment was carried out with distilled water. Twenty-

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SALTS USED		CONCENTRATIONS										CONTROL
		M 2	M 10	M 20	M 100	M 200	M 1000	M 2000	M 10000			
Magnesium sulphate	Length of leaf*	20	40	60	80	83	112	97	90	80		
	Length of root	35	65	65	80	85	140	118	100	125		
	Number of roots	1	1	1	3	3	9	6	5	6		
Magnesium chloride	Length of leaf	died	27	56	80	83	93	100	88	80		
	Length of root	died	42	45	70	90	165	110	112	125		
	Number of roots	died	1	1	2	2	6	8	6	6		
Calcium chloride	Length of leaf	died	30	47	73	80	90	90	105	80		
	Length of root	died	25	40	97	100	150	100	150	125		
	Number of roots	died	3	6	6	6	8	9	7	6		
Sodium sulphate	Length of leaf	died	33	42	120	107	100	97	93	80		
	Length of root	died	30	40	90	85	90	100	100	125		
	Number of roots	died	1	6	7	7	9	7	9	6		
Sodium chloride	Length of leaf	died	42	67	97	95	85	82	82	80		
	Length of root	died	30	63	150	130	70	110	130	125		
	Number of roots	died	3	6	5	5	4	7	7	6		
Sodium carbonate	Length of leaf	died	died	33	83	117	117	97	85	80		
	Length of root	died	died	20	30	145	145	105	85	125		
	Number of roots	died	died	1	5	5	6	7	7	6		
Sodium bicarbonate	Length of leaf	died	30	40	105	115	110	103	103	80		
	Length of root	died	18	45	90	140	60	90	105	125		
	Number of roots	died	1	1	6	5	6	6	7	6		

* Lengths of leaf and root are expressed in millimeters.

five seedlings were grown in each culture at ordinary temperature and the evaporated water was supplemented with distilled water from time to time to keep the solutions at their original concentrations. After ten days, the difference of development was very striking, and then the determinations recorded in the table on p. 238 were made:

The results show that each salt acted as a toxic agent or a stimulant upon the growth of rice seedlings, according to its concentrations. Magnesium sulphate and chloride, calcium chloride and sodium carbonate were injurious when the concentrations were greater than about $\frac{M}{200}$, while sodium sulphate, chloride and bicarbonate were toxic when the concentrations were greater than $\frac{M}{100}$. In every salt, when the concentration was such that the toxic action ceased, the stimulating effect began and attained its highest degree in the following order of concentration: magnesium sulphate $\frac{M}{1000}$, magnesium chloride $\frac{M}{2000}$, calcium chloride $\frac{M}{1000}$, sodium sulphate $\frac{M}{100}$, sodium chloride $\frac{M}{100}$, sodium carbonate $\frac{M}{200}$ to $\frac{M}{1000}$ and sodium bicarbonate $\frac{M}{200}$.

Experiment II.

On June 13, 1912, twenty-five rice seeds of almost uniform size and specific gravity (1.158–1.185) were sown in the beakers, about 55 cm. in diameter and 7 cm. deep, each containing 30 cc. of solution of each salt while distilled water served as control. The concentration of the salts is indicated in the table. The beakers were kept in a room of normal temperature, and evaporated water was supplemented with distilled water from time to time to keep the solutions always in their initial concentrations. After thirty-six days, the difference of their development was very striking. The measurements recorded on the following page were then made:

It is assumed that the plant is adversely affected by the salts, if the length of root be half that of the control plants, even though the length of leaf be greater than that of the control leaf.

In this case as in the previous experiment, the growth of the seedlings was injured or stimulated by each salt according to the concentration. In the concentration at which the toxic action ceases, the stimulating action began and attained its highest point at certain definite dilutions. The growth was injured by mag-

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SALTS USED	N 2	N 5	CONCENTRATIONS								CONTROL
			N 10	N 50	N 100	N 500	N 1000	N 5000			
Magnesium sulphate	Only 18 seeds germinated	22	30	85	100	130	110	120	100	100	
	Length of leaf*								115	6	
	Length of root								100	6	
Magnesium chloride	Number of roots			1	4	6	6	6	6	6	
	Length of leaf	All seeds germinated but not one developed	25	83	105	100	105	107	100	100	
	Length of root								115	6	
Calcium chloride	Number of roots			3	3	6	6	6	6	6	
	Length of leaf	Only 6 seeds germinated	18	85	105	110	145	95	100	100	
	Length of root								115	6	
Sodium sulphate	Number of roots			5	6	6	6	6	6	6	
	Length of leaf	12	23	150	165	145	120	100	100	100	
	Length of root								115	6	
Sodium chloride	Number of roots			4	4	6	7	7	6	6	
	Length of leaf	4	35	155	137	110	110	110	100	100	
	Length of root								115	6	
Sodium carbonate	Number of roots			6	6	6	7	7	6	6	
	Length of leaf	Only 5 seeds germinated	5	85	120	120	110	110	100	100	
	Length of root								115	6	
Sodium bicarbonate	Number of roots			6	7	7	7	7	6	6	
	Length of leaf	17	55	95	120	125	100	110	100	100	
	Length of root								115	6	
	Number of roots			4	5	6	6	7	6	6	
	Length of leaf								100	100	
	Length of root								115	6	

*Lengths of leaf and root are expressed in millimeters.

nesium sulphate in concentration greater than $\frac{N}{100}$ and highly stimulated by $\frac{N}{500}$. Magnesium chloride was also toxic in concentration greater than $\frac{N}{100}$ and attained highest stimulating point in concentration of $\frac{N}{5000}$. The toxic concentration of calcium chloride, sodium sulphate, sodium chloride, sodium carbonate and bicarbonate in each case was greater than $\frac{N}{100}$, $\frac{N}{100}$, $\frac{N}{100}$, $\frac{N}{100}$, $\frac{N}{100}$ respectively and highest stimulation was reached in dilution of $\frac{N}{1000}$, $\frac{N}{100}$, $\frac{N}{100}$, $\frac{N}{500}$ and $\frac{N}{500}$ respectively.

For convenience of comparison, the concentration of toxicity and stimulation of the seven salts in the two experiments are brought together in the following table.

SALTS USED	CONCENTRATION OF TOXICITY		DILUTION OF HIGHEST STIMULATION	
	Experiment I	Experiment II	Experiment I	Experiment II
	greater than	greater than		
Magnesium sulphate...	$\frac{M}{100}$ ($\frac{N}{50}$)	$\frac{N}{100}$	$\frac{M}{1000}$ ($\frac{N}{500}$)	$\frac{N}{500}$
Magnesium chloride...	$\frac{M}{100}$ ($\frac{N}{50}$)	$\frac{N}{100}$	$\frac{M}{2000}$ ($\frac{N}{1000}$)	$\frac{N}{5000}$
Calcium chloride	$\frac{M}{200}$ ($\frac{N}{100}$)	$\frac{N}{100}$	$\frac{M}{10000}$ ($\frac{N}{5000}$)	$\frac{N}{1000}$
Sodium sulphate	$\frac{M}{100}$ ($\frac{N}{50}$)	$\frac{N}{50}$	$\frac{M}{100}$ ($\frac{N}{50}$)	$\frac{N}{100}$
Sodium chloride	$\frac{M}{100}$ ($\frac{N}{100}$)	$\frac{N}{100}$	$\frac{M}{100}$ ($\frac{N}{100}$)	$\frac{N}{100}$
Sodium carbonate	$\frac{M}{100}$ ($\frac{N}{50}$)	$\frac{N}{100}$	$\frac{M}{200}$ to $\frac{M}{1000}$ ($\frac{N}{100}$ to $\frac{N}{500}$)	$\frac{N}{500}$
Sodium bicarbonate....	$\frac{M}{100}$ ($\frac{N}{50}$)	$\frac{N}{50}$	$\frac{M}{200}$ ($\frac{N}{100}$)	$\frac{N}{500}$

As seen in the table, both results almost coincide on the toxic and stimulating point. A slight fluctuation of these points is probably due to the fact that the plant growth varies to a certain extent with the temperature and other factors, since these experiments were not carried on at constant temperature and under identical conditions.

Conclusions.

From these two experiments we may safely conclude as follows:

1. The alkali salts under examination act as agents both toxic and stimulating upon the growth of rice seedlings, according to their concentrations.
2. The toxic concentrations of magnesium sulphate and chloride, calcium chloride, sodium chloride and carbonate are greater

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than $\frac{N}{100}$ while sodium sulphate and bicarbonate are greater than $\frac{N}{50}$.

3. The highest stimulation is observed in the dilution of $\frac{N}{50}$ for magnesium sulphate, $\frac{N}{1000}$ to $\frac{N}{5000}$ for magnesium chloride, $\frac{N}{1000}$ to $\frac{N}{5000}$ for calcium chloride, $\frac{N}{50}$ to $\frac{N}{100}$ for sodium chloride, $\frac{N}{100}$ to $\frac{N}{500}$ for sodium carbonate and bicarbonate.

PART II. ON THE ANTAGONISM BETWEEN THE TOXIC EFFECTS OF TWO SALTS UPON THE GROWTH OF RICE SEEDLINGS.

The results of experiments with a single salt solution have been reported in the preceding section, but they cannot be correlated with our knowledge of alkali soils, since, as Kearney and Cameron⁶ pointed out, in nature we have always to do with a mixture of salts and never with single solutions. They found, as in Loeb's striking experiments with marine animals, that by adding sodium salts to the solution of magnesium salts the critical concentrations of the latter could be raised considerably. In the case of *Lupinus albus* and *Medicago sativa*, the neutralizing effect became enormous when salts of calcium were added to the solutions of sulphate and chlorides of magnesium and sodium.

The physiology of the decreasing toxicity of a salt due to the presence of a second salt in the solution, was specially discussed by Osterhout⁷ from the view-point of Loeb's conception of a "physiologically balanced salts solution." It has been shown that marine plants as well as marine animals are very sensitive to pure salt solutions, but thrive well in solutions containing a mixture of salts, even though each component is present in an amount that is toxic in pure solution. A mixture of the more important salts present in sea water, each at about the concentration at which it occurs in the sea, was found to be the best medium for the growth of marine algae. The same phenomenon has been observed in the case of land plants.

Kearney and Harter⁸ investigated the neutralizing effect of calcium sulphate upon the toxicity of magnesium and sodium salts with eight

⁶ Bulletin No. 71, Bureau of Plant Industry, U. S. Dept. of Agriculture, 1912.

⁷ This *Journal*, i, pp. 363-369, 1906; *Bot. Gaz.*, xlii, pp. 127-134, 1906; *Univ. of Calif. Pub. Bot.*, ii, p. 317, 1907; *Jahrb. f. wiss. Bot.* xvi, p. 121, 1908; *Bot. Gaz.*, xlv, p. 117, 1908; *Univ. of Calif. Pub. Bot.*, iii, pp. 331-337, 1908; *Bot. Gaz.*, xlviii, pp. 98-104, 1909.

⁸ Bulletin No. 113, Bureau of Plant Industry, U. S. Dept. of Agriculture, 1907.

different land plants and found that the presence of calcium sulphate tends greatly to diminish, not only the differences between different species as to their tolerance of magnesium and sodium salts, but also the differences between the latter in their toxicity to the same species. The neutralizing effect of calcium sulphate is generally much more marked with magnesium than with sodium salts.

In 1907, Benecke⁹ studied the poisonous action of various salts upon the growth of spirogyra. The result of his investigation was summarized as follows: Chloride, nitrate, sulphate and phosphate of sodium, potassium, magnesium and iron are more or less poisonous, and among these cations iron and magnesium are more poisonous than potassium, sodium is less poisonous than potassium; among the anions, chlorine is least poisonous. The toxicity of these anions and cations can be neutralized or decreased by the addition of calcium ions. Loew and Aso¹⁰ also studied the same subject in relation to spirogyra and observed that calcium salts can prevent the toxic effects of magnesium salts while potassium salts can retard but not entirely prevent the injurious action of the same.

Takeuchi¹¹ has pointed out, at the end of his investigation on the behavior of algae to salts at certain concentrations, that the injurious action of magnesium salts can only completely be overcome by calcium salts, and not by sodium or potassium salts. This has been observed not only with algae, but also with young plants of barley and maize which were deprived of their endosperm.

Hansteen¹² has recently investigated the antagonism between cations upon the growth of wheat seedlings and shown that the pure solutions of potassium, sodium and magnesium salts are more or less injurious according to their concentrations. But in combination with calcium salts, their injurious effect on the growth of leaves, roots and root-hairs is greatly decreased.

Toxic and antagonistic effects of salts as related to ammonia formation by *Bacillus subtilis* were also investigated by Lipman¹³ and the following conclusions were reported: 1. Each of the four chlorides (CaCl_2 , MgCl_2 , KCl , NaCl) is toxic for *Bacillus subtilis*, in the order given, the first being the most toxic and the fourth the least. This is different from the results with higher plants, where magnesium is the most toxic and calcium the least. 2. A marked antagonism exists between calcium and potassium, magnesium and sodium, potassium and sodium. 3. No antagonism exists between magnesium and calcium but the toxic effect of each is increased by combination with the other. This is just the opposite of what has hitherto been found for plants.

⁹ *Ber. d. bot. Gesellschaft.*, xxv, p. 322, 1907,

¹⁰ *Bull. Coll. Agric.*, Tokyo Imp. Univ., vii, pp. 395-409, 1906-08.

¹¹ *Ibid.*, vii, p. 628, 1906-08.

¹² *Nyt. Mag. Naturvidensk.*, xlvii, pp. 181-192, 1909; ref. *Exp. Sta. Rec.*,

U. S. Dept. of Agriculture, xxiii, p. 28, 1910.

¹³ *Bot. Gaz.*, xlviii, pp. 105-124, 1909.

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As above stated, it is clear that the toxicity of a single salt solution may be neutralized by the presence of a second salt, especially calcium salts. It was desirable to investigate the influence of salts common in alkali soils upon the growth of rice plants. We have therefore selected chloride of sodium, magnesium and calcium, and sulphate of sodium and magnesium as the salts to be tested and have examined the respective antagonisms between these salts in combination.

I. *Experiment with NaCl and MgCl₂.*

The antagonism between sodium and magnesium chloride was established with young rice seedlings, about 10 mm. high, which were grown in distilled water from seeds of almost uniform size and specific gravity (1.185-1.200). Beakers of about 5.5 cm. in diameter and 7 cm. deep, each containing the solutions noted in the table¹⁴ were used for the experiment, the seedlings being placed in the culture fluids on November 19 (1912). Five seedlings were grown in each culture in the greenhouse and the evaporated water was supplemented with distilled water from time to time to keep the solutions at the initial dilutions. After twelve days, the difference in development in the respective cultures was very remarkable. The following determinations were made:

SOLUTIONS USED		LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
		mm.	mm.	
$\frac{N}{10}$ NaCl, 30 cc.....		38	25	1
$\frac{N}{10}$ NaCl, 25 cc. + $\frac{N}{10}$ MgCl ₂ , 5 cc.....		57	40	6*
$\frac{N}{10}$ NaCl, 20 cc. + $\frac{N}{10}$ MgCl ₂ , 10 cc.....		53	30	4*
$\frac{N}{10}$ NaCl, 15 cc. + $\frac{N}{10}$ MgCl ₂ , 15 cc.....		50	32	3*
$\frac{N}{10}$ NaCl, 10 cc. + $\frac{N}{10}$ MgCl ₂ , 20 cc.....		45	28	2*
$\frac{N}{10}$ NaCl, 5 cc. + $\frac{N}{10}$ MgCl ₂ , 25 cc.....		45	20	1
$\frac{N}{10}$ MgCl ₂ , 30 cc.....		42	20	1
Distilled water, 30 cc.....		50	80	6

*Only one root was well developed.

¹⁴ As already proved in the previous section, a pure solution of each salt to be tested is very injurious to the growth of rice seedlings in the concentration of $\frac{N}{10}$.

From these results, it is clear that the poisonous effect of sodium and magnesium chloride largely disappears when we mix the two salts in favorable proportions. This phenomenon is due to the antagonism between sodium and magnesium ions, since the anions were similar in both salts. In these favorable mixtures, the length of leaf became greater than that in distilled water, but the length of roots and the number of roots was invariably less than in the case of the control plants. It is evident therefore that the toxic effect of sodium and of magnesium ions was mutually counteracted but not completely neutralized. And it is further evident that the toxic effect of the sodium ion was antagonized much more by magnesium ion than the latter by the former, an observation which coincides with the results of Osterhout obtained with wheat seedlings.

II. *Experiment with Na₂SO₄ and MgSO₄.*

The antagonistic action of sodium and magnesium ions on each other was once more tested with sodium and magnesium sulphate by exactly the same method as in the preceding experiment, except that the young seedlings transplanted were about 20 mm. in height at the beginning of the experiment. The results obtained were as follows:

SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ Na ₂ SO ₄ , 30 cc.....	45	35	1
$\frac{N}{10}$ Na ₂ SO ₄ , 25 cc. + $\frac{N}{10}$ MgSO ₄ , 5 cc.....	60	40	5*
$\frac{N}{10}$ Na ₂ SO ₄ , 20 cc. + $\frac{N}{10}$ MgSO ₄ , 10 cc.....	55	30	5*
$\frac{N}{10}$ Na ₂ SO ₄ , 15 cc. + $\frac{N}{10}$ MgSO ₄ , 15 cc.....	55	35	4*
$\frac{N}{10}$ Na ₂ SO ₄ , 10 cc. + $\frac{N}{10}$ MgSO ₄ , 20 cc.....	55	40	3*
$\frac{N}{10}$ Na ₂ SO ₄ , 5 cc. + $\frac{N}{10}$ MgSO ₄ , 25 cc.....	50	33	1
$\frac{N}{10}$ MgSO ₄ , 30 cc.....	40	20	1
Distilled water, 30 cc.....	80	50	7

* Only one root was well developed.

In this case, we also observed that the mutual counteraction between sodium and magnesium ions was clearly revealed, though they did not perfectly neutralize each other. The neutralizing power of magnesium ion toward the toxic effect of sodium ion

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was greater than that of sodium to magnesium, for in the case of $\frac{N}{10}$ Na_2SO_4 , 25 cc., + $\frac{N}{10}$ MgSO_4 , 5 cc., the highest development of the seedlings was observed. The result of this experiment almost coincides with that of the preceding one.

III. *Experiment with NaCl and CaCl₂.*

The antagonistic phenomenon between sodium and calcium ions was examined with $\frac{N}{10}$ solution of sodium and calcium chloride in manner identical with that followed in the case of the experiment with sodium and magnesium chloride. Twelve days after the seedlings were transplanted to the respective culture solutions, they showed very remarkable differences of development. The plants were measured on that day with the following result.

SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ NaCl, 30 cc.....	38	25	1
$\frac{N}{10}$ NaCl, 25 cc. + $\frac{N}{10}$ CaCl ₂ , 5 cc.....	69	60	9
$\frac{N}{10}$ NaCl, 20 cc. + $\frac{N}{10}$ CaCl ₂ , 10 cc.....	55	50	7
$\frac{N}{10}$ NaCl, 15 cc. + $\frac{N}{10}$ CaCl ₂ , 15 cc.....	47	35	5
$\frac{N}{10}$ NaCl, 10 cc. + $\frac{N}{10}$ CaCl ₂ , 20 cc.....	47	40	5
$\frac{N}{10}$ NaCl, 5 cc. + $\frac{N}{10}$ CaCl ₂ , 25 cc.....	47	35	3
$\frac{N}{10}$ CaCl ₂ , 30 cc.....	40	35	1
Distilled water, 30 cc.....	50	80	6

It is evident that in a mixture of sodium and calcium ions in proper proportion, each of which individually is poisonous, the toxic effect of these ions is almost mutually counteracted and a medium is produced in which the plant may live almost indefinitely. The toxic effect of sodium ion almost completely disappeared when we added a little calcium ion; on the other hand, the poisonous effect of calcium ion was excluded by the addition of a great amount of sodium ion.

IV. *Experiment with MgCl₂ and CaCl₂.*

On July 20, 1912, thirty seeds of rice which were almost uniform in size and specific gravity (1.185-1.200) were sown in beakers of about 5.5 cm. diameter and 7 cm. deep, each containing

the solutions noted in the table. The beakers were kept at room temperature and covered with glass plates to exclude dust and retard evaporation until the seedlings reached a height of about 15 mm. The evaporated water was supplemented with distilled water from time to time to keep the culture media at their initial concentrations. The difference in the development of the plants became very marked. On August 20, the plants were measured, and the results obtained are shown in the following table:

SOLUTIONS USED	LENGTH OF	LENGTH OF	NUMBER OF ROOTS
	LEAF	ROOT	
	mm.	mm.	
$\frac{N}{10}$ CaCl ₂ , 30 cc.....	35	8	1
$\frac{N}{10}$ CaCl ₂ , 25 cc. + $\frac{N}{10}$ MgCl ₂ , 5 cc.....	55	5	2
$\frac{N}{10}$ CaCl ₂ , 20 cc. + $\frac{N}{10}$ MgCl ₂ , 10 cc.....	80	6	5
$\frac{N}{10}$ CaCl ₂ , 15 cc. + $\frac{N}{10}$ MgCl ₂ , 15 cc.....	95	25	4
$\frac{N}{10}$ CaCl ₂ , 10 cc. + $\frac{N}{10}$ MgCl ₂ , 20 cc.....	105	22	5
$\frac{N}{10}$ CaCl ₂ , 5 cc. + $\frac{N}{10}$ MgCl ₂ , 25 cc.....	110	40	8
$\frac{N}{10}$ MgCl ₂ , 30 cc.....	45	5	1

From the above table, it is clear that in a mixture of calcium and magnesium ions the toxic effects of these cations were mutually counteracted. The amount of calcium required to antagonize the toxic effect of magnesium was less than that of the latter to the former, for we observed that the highest development of the plants was attained in the mixture of $\frac{N}{10}$ CaCl₂, 5 cc., + $\frac{N}{10}$ MgCl₂, 25 cc.; consequently the antagonizing power of calcium is strong and that of magnesium is weak.

V. *Experiment with NaCl and Na₂SO₄.*

In the above four experiments, we examined the antagonisms between the metallic ions in regard to their toxic effects upon the growth of rice seedlings. We then undertook to investigate the question of the mutual power of counteracting injurious effects of anions upon the development of rice plants. Hence, sodium chloride and sulphate were selected as salts to be tested and examined in a manner similar to that followed in the case of the experiment with sodium and magnesium chloride. The plants were measured as follows:

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SOLUTIONS USED		LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
		mm.	mm.	
$\frac{N}{10}$	NaCl, 30 cc.....	42	30	1
$\frac{N}{10}$	NaCl, 25 cc. + $\frac{N}{10}$ Na ₂ SO ₄ , 5 cc.....	60	40	1
$\frac{N}{10}$	NaCl, 20 cc. + $\frac{N}{10}$ Na ₂ SO ₄ , 10 cc.....	53	30	1
$\frac{N}{10}$	NaCl, 15 cc. + $\frac{N}{10}$ Na ₂ SO ₄ , 15 cc.....	42	30	1
$\frac{N}{10}$	NaCl, 10 cc. + $\frac{N}{10}$ Na ₂ SO ₄ , 20 cc.....	52	30	1
$\frac{N}{10}$	NaCl, 5 cc. + $\frac{N}{10}$ Na ₂ SO ₄ , 25 cc.....	55	30	1
$\frac{N}{10}$	Na ₂ SO ₄ , 30 cc.....	45	35	1
	Distilled water, 30 cc.....	80	50	7

The counteraction observed in this experiment is doubtless due to the actions between the anions (Cl' and SO₄'') present in the culture media since the cations in both salts are the same. The ratio of these anions required to produce the most favorable medium for the development of the plants was 25:5, although the development of the seedling did not reach that of the control plants. The antagonistic power of the SO₄'' ion required to neutralize the toxic effect of Cl' ion was slightly greater than that of Cl' to the SO₄'' ion.

VI. Experiment with MgCl₂ and MgSO₄.

The antagonism between Cl' and SO₄'' ions was again examined with magnesium chloride and sulphate in the same manner as in the preceding experiment. The following result which is similar to that of the experiment with sodium chloride and sulphate was obtained.

SOLUTIONS USED		LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
		mm.	mm.	
$\frac{N}{10}$	MgCl ₂ , 30 cc.....	45	35	1
$\frac{N}{10}$	MgCl ₂ , 25 cc. + $\frac{N}{10}$ MgSO ₄ , 5 cc.....	60	40	1
$\frac{N}{10}$	MgCl ₂ , 20 cc. + $\frac{N}{10}$ MgSO ₄ , 10 cc.....	55	30	1
$\frac{N}{10}$	MgCl ₂ , 15 cc. + $\frac{N}{10}$ MgSO ₄ , 15 cc.....	48	30	1
$\frac{N}{10}$	MgCl ₂ , 10 cc. + $\frac{N}{10}$ MgSO ₄ , 20 cc.....	50	25	1
$\frac{N}{10}$	MgCl ₂ , 5 cc. + $\frac{N}{10}$ MgSO ₄ , 25 cc.....	55	25	1
$\frac{N}{10}$	MgSO ₄ , 30 cc.....	40	25	1
	Distilled water, 30 cc.....	80	50	7

VII. *Experiment with NaCl and MgSO₄.*

The antagonistic action of Na⁺, Mg⁺⁺, Cl⁻, and SO₄⁼⁼ ions on each other was established with sodium chloride and magnesium sulphate in the same manner as in the case of Experiment I.

The following result was obtained.

SOLUTIONS USED		LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
		mm.	mm.	
$\frac{N}{10}$ NaCl, 30 cc.....	$\frac{N}{10}$	38	25	1
$\frac{N}{10}$ NaCl, 25 cc. + $\frac{N}{10}$ MgSO ₄ , 5 cc.....	$\frac{N}{10}$	55	37	6*
$\frac{N}{10}$ NaCl, 20 cc. + $\frac{N}{10}$ MgSO ₄ , 10 cc.....	$\frac{N}{10}$	50	33	4*
$\frac{N}{10}$ NaCl, 15 cc. + $\frac{N}{10}$ MgSO ₄ , 15 cc.....	$\frac{N}{10}$	45	30	3*
$\frac{N}{10}$ NaCl, 10 cc. + $\frac{N}{10}$ MgSO ₄ , 20 cc.....	$\frac{N}{10}$	48	31	1
$\frac{N}{10}$ NaCl, 5 cc. + $\frac{N}{10}$ MgSO ₄ , 25 cc.....	$\frac{N}{10}$	48	32	1
$\frac{N}{10}$ MgSO ₄ , 30 cc.....	$\frac{N}{10}$	30	25	1
Distilled water, 30 cc.....		50	80	6

* Only one root was well developed.

From the table, it is clear that the observed antagonistic action between these ions almost coincides with the results of Experiments I and V or VI.

VIII. *Experiment with Na₂SO₄ and MgCl₂.*

The same antagonism as in the preceding experiment was again examined with sodium sulphate and magnesium chloride as before. The result obtained almost coincides with that of the preceding experiment as will be seen in the following table.

SOLUTIONS USED		LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
		mm.	mm.	
$\frac{N}{10}$ Na ₂ SO ₄ , 30 cc.....	$\frac{N}{10}$	45	35	1
$\frac{N}{10}$ Na ₂ SO ₄ , 25 cc. + $\frac{N}{10}$ MgCl ₂ , 5 cc.....	$\frac{N}{10}$	80	40	6*
$\frac{N}{10}$ Na ₂ SO ₄ , 20 cc. + $\frac{N}{10}$ MgCl ₂ , 10 cc.....	$\frac{N}{10}$	80	38	6*
$\frac{N}{10}$ Na ₂ SO ₄ , 15 cc. + $\frac{N}{10}$ MgCl ₂ , 15 cc.....	$\frac{N}{10}$	65	45	4*
$\frac{N}{10}$ Na ₂ SO ₄ , 10 cc. + $\frac{N}{10}$ MgCl ₂ , 20 cc.....	$\frac{N}{10}$	50	30	1
$\frac{N}{10}$ Na ₂ SO ₄ , 5 cc. + $\frac{N}{10}$ MgCl ₂ , 25 cc.....	$\frac{N}{10}$	60	25	1
$\frac{N}{10}$ MgCl ₂ , 30 cc.....	$\frac{N}{10}$	55	40	1
Distilled water, 30 cc.....		80	50	7

* Only one root was well developed.

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IX. Experiment with Na_2SO_4 and CaCl_2 .

The antagonistic action of Na^+ , Ca^{++} , Cl' and SO_4'' on each other was examined with sodium sulphate and calcium chloride in the same manner as in Experiment II. The result obtained was as follows:

SOLUTIONS USED		LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
		mm.	mm.	
$\frac{N}{10}$	Na_2SO_4 , 30 cc.....	45	35	1
$\frac{N}{10}$	Na_2SO_4 , 25 cc. + $\frac{N}{10}$ CaCl_2 , 5 cc.....	80	90	9
$\frac{N}{10}$	Na_2SO_4 , 20 cc. + $\frac{N}{10}$ CaCl_2 , 10 cc.....	80	90	7
$\frac{N}{10}$	Na_2SO_4 , 15 cc. + $\frac{N}{10}$ CaCl_2 , 15 cc.....	70	50	7
$\frac{N}{10}$	Na_2SO_4 , 10 cc. + $\frac{N}{10}$ CaCl_2 , 20 cc.....	57	30	5
$\frac{N}{10}$	Na_2SO_4 , 5 cc. + $\frac{N}{10}$ CaCl_2 , 25 cc.....	50	38	4
$\frac{N}{10}$	CaCl_2 , 30 cc.....	45	25	1
	Distilled water, 30 cc.....	80	50	7

As will be seen in the above result, in a suitable mixture of Na^+ , Ca^{++} , Cl' and SO_4'' ions, their toxic effects completely disappear. It was also observed that the combined antagonistic actions of cations and anions have a more favorable effect than that of one of them.

X. Experiment with MgSO_4 and CaCl_2 .

The antagonistic action of Mg^{++} , Ca^{++} , Cl' and SO_4'' ions on each other was established with magnesium sulphate and calcium chloride as in Experiment IV. The following result which is similar to that of the preceding experiment was obtained.

SOLUTIONS USED		LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
		mm.	mm.	
$\frac{N}{10}$	MgSO_4 , 30 cc.....	50	23	1
$\frac{N}{10}$	MgSO_4 , 25 cc. + $\frac{N}{10}$ CaCl_2 , 5 cc.....	135	55	4
$\frac{N}{10}$	MgSO_4 , 20 cc. + $\frac{N}{10}$ CaCl_2 , 10 cc.....	135	50	6
$\frac{N}{10}$	MgSO_4 , 15 cc. + $\frac{N}{10}$ CaCl_2 , 15 cc.....	120	45	6
$\frac{N}{10}$	MgSO_4 , 10 cc. + $\frac{N}{10}$ CaCl_2 , 20 cc.....	85	25	4
$\frac{N}{10}$	MgSO_4 , 5 cc. + $\frac{N}{10}$ CaCl_2 , 25 cc.....	80	18	3
$\frac{N}{10}$	CaCl_2 , 30 cc.....	35	8	1

Conclusions.

The results obtained in all of these experiments, may be summarized as follows:

1. The salts under examination, used separately, are very poisonous in $\frac{N}{10}$ concentration upon the growth of the rice plant, but when the two salts are mixed with each other in a suitable proportion, the toxic effect of each salt more or less completely disappears. This result is of great importance in alkali soil investigations.

2. The antagonistic action of salts is due to that of the ions formed by the dissociation of the salt.

3. In general, divalent cations are markedly antagonized by monovalent cations, but on the other hand, monovalent cations do not strongly antagonize divalent cations.

4. Among the divalent cations, calcium shows a more marked antagonism than magnesium.

5. The antagonism between Cl' and SO_4'' , though it is small in comparison with that between cations, is also present in no slight degree.

PART III. ON THE ANTAGONISTIC ACTION OF SODIUM AND POTASSIUM SALTS.

In Part III we have specially undertaken to test the antagonism between sodium salts, potassium salts, and sodium and potassium salts. Chloride, sulphate and nitrate of soda and potash were selected as the salts to be tested, and the following experiments were made:

I. *Experiment with Na_2SO_4 and K_2SO_4 .*

Eight beakers of about 5.5 cm. in diameter and 7 cm. deep each containing 30 cc. of culture fluids, of the composition noted in the table, served for the experiment. One beaker containing distilled water served as control. On February 1, 1913, young rice seedlings which were grown in distilled water, were transplanted into the beakers, each receiving five healthy individuals of uniform size (about 20 mm. long) and kept in a greenhouse.

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The evaporated water was replaced with distilled water from time to time. A decided difference in plant growth was noticed from day to day. On February 18, measurements were made with the following result:

SOLUTIONS USED		LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
		mm.	mm.	
$\frac{N}{10}$	Na_2SO_4 , 30 cc.....	35	35	2*
$\frac{N}{10}$	Na_2SO_4 , 25 cc. + $\frac{N}{10}$ K_2SO_4 , 5 cc.....	67	43	6*
$\frac{N}{10}$	Na_2SO_4 , 20 cc. + $\frac{N}{10}$ K_2SO_4 , 10 cc.....	47	37	6*
$\frac{N}{10}$	Na_2SO_4 , 15 cc. + $\frac{N}{10}$ K_2SO_4 , 15 cc.....	47	37	6*
$\frac{N}{10}$	Na_2SO_4 , 10 cc. + $\frac{N}{10}$ K_2SO_4 , 20 cc.....	47	35	6*
$\frac{N}{10}$	Na_2SO_4 , 5 cc. + $\frac{N}{10}$ K_2SO_4 , 25 cc.....	50	43	7*
$\frac{N}{10}$	K_2SO_4 , 30 cc.....	40	37	6*
	Distilled water, 30 cc.....	68	43	7

* Only one root was well developed.

II. Experiment with NaCl and KCl.

The antagonistic action of sodium and potassium on each other was again established with sodium and potassium chloride in the same manner as in the preceding experiment:

SOLUTIONS USED		LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
		mm.	mm.	
$\frac{N}{10}$	NaCl , 30 cc.....	35	35	2*
$\frac{N}{10}$	NaCl , 25 cc. + $\frac{N}{10}$ KCl , 5 cc.....	60	45	6*
$\frac{N}{10}$	NaCl , 20 cc. + $\frac{N}{10}$ KCl , 10 cc.....	55	45	6*
$\frac{N}{10}$	NaCl , 15 cc. + $\frac{N}{10}$ KCl , 15 cc.....	55	40	6*
$\frac{N}{10}$	NaCl , 10 cc. + $\frac{N}{10}$ KCl , 20 cc.....	42	45	7*
$\frac{N}{10}$	NaCl , 5 cc. + $\frac{N}{10}$ KCl , 25 cc.....	50	35	6*
$\frac{N}{10}$	KCl , 30 cc.....	40	25	6*
	Distilled water, 30 cc.....	68	43	7

* Only one root was well developed.

III. Experiment with NaNO_3 and KNO_3 .

The antagonistic action of sodium and potassium on each other was once more tested with sodium and potassium nitrate and the following result was obtained:

SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ NaNO ₃ , 30 cc.....	35	35	2*
$\frac{N}{10}$ NaNO ₃ , 25 cc. + $\frac{N}{10}$ KNO ₃ , 5 cc.....	60	37	7*
$\frac{N}{10}$ NaNO ₃ , 20 cc. + $\frac{N}{10}$ KNO ₃ , 10 cc.....	50	40	6*
$\frac{N}{10}$ NaNO ₃ , 15 cc. + $\frac{N}{10}$ KNO ₃ , 15 cc.....	50	35	7*
$\frac{N}{10}$ NaNO ₃ , 10 cc. + $\frac{N}{10}$ KNO ₃ , 20 cc.....	43	35	7*
$\frac{N}{10}$ NaNO ₃ , 5 cc. + $\frac{N}{10}$ KNO ₃ , 25 cc.....	50	35	7*
$\frac{N}{10}$ KNO ₃ , 30 cc.....	40	35	4*
Distilled water, 30 cc.....	68	43	7

* Only one root was well developed.

IV. Experiment with K₂SO₄ and KCl.

The antagonism between SO₄'' and Cl' ions was already observed in Experiment No. V of Part II with sodium sulphate and chloride. This was then once more examined with potassium sulphate and chloride in the same manner as in Experiment I, and the result, which coincides with that of the experiment with sodium salts, is given in the following table:

SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ K ₂ SO ₄ , 30 cc.....	40	35	6*
$\frac{N}{10}$ K ₂ SO ₄ , 25 cc. + $\frac{N}{10}$ KCl, 5 cc.....	47	43	7*
$\frac{N}{10}$ K ₂ SO ₄ , 20 cc. + $\frac{N}{10}$ KCl, 10 cc.....	47	35	6*
$\frac{N}{10}$ K ₂ SO ₄ , 15 cc. + $\frac{N}{10}$ KCl, 15 cc.....	40	32	6*
$\frac{N}{10}$ K ₂ SO ₄ , 10 cc. + $\frac{N}{10}$ KCl, 20 cc.....	50	50	7*
$\frac{N}{10}$ K ₂ SO ₄ , 5 cc. + $\frac{N}{10}$ KCl, 25 cc.....	47	35	7*
$\frac{N}{10}$ KCl, 30 cc.....	35	25	6*
Distilled water, 30 cc.....	68	43	7

* Only one root was well developed.

V. Experiment with Na₂SO₄ and NaNO₃.

The antagonism between SO₄'' and NO₃' ions on each other was established with sodium sulphate and nitrate as in Experiment I. The following result was obtained:

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SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ Na ₂ SO ₄ , 30 cc.....	35	35	2*
$\frac{N}{10}$ Na ₂ SO ₄ , 25 cc. + $\frac{N}{10}$ NaNO ₃ , 5 cc.....	52	35	5*
$\frac{N}{10}$ Na ₂ SO ₄ , 20 cc. + $\frac{N}{10}$ NaNO ₃ , 10 cc.....	52	35	5*
$\frac{N}{10}$ Na ₂ SO ₄ , 15 cc. + $\frac{N}{10}$ NaNO ₃ , 15 cc.....	40	35	5*
$\frac{N}{10}$ Na ₂ SO ₄ , 10 cc. + $\frac{N}{10}$ NaNO ₃ , 20 cc.....	50	35	5*
$\frac{N}{10}$ Na ₂ SO ₄ , 5 cc. + $\frac{N}{10}$ NaNO ₃ , 25 cc.....	45	30	5*
$\frac{N}{10}$ NaNO ₃ , 30 cc.....	35	35	2*
Distilled water, 30 cc.....	68	43	7

* Only one root was well developed.

VI. Experiment with K₂SO₄ and KNO₃.

An experiment similar to the preceding one was made with potassium salts and a similar result was obtained, as will be seen in the following table:

SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ K ₂ SO ₄ , 30 cc.....	40	35	6*
$\frac{N}{10}$ K ₂ SO ₄ , 25 cc. + $\frac{N}{10}$ KNO ₃ , 5 cc.....	58	45	7*
$\frac{N}{10}$ K ₂ SO ₄ , 20 cc. + $\frac{N}{10}$ KNO ₃ , 10 cc.....	53	38	6*
$\frac{N}{10}$ K ₂ SO ₄ , 15 cc. + $\frac{N}{10}$ KNO ₃ , 15 cc.....	45	38	6*
$\frac{N}{10}$ K ₂ SO ₄ , 10 cc. + $\frac{N}{10}$ KNO ₃ , 20 cc.....	50	42	6*
$\frac{N}{10}$ K ₂ SO ₄ , 5 cc. + $\frac{N}{10}$ KNO ₃ , 25 cc.....	40	35	6*
$\frac{N}{10}$ KNO ₃ , 30 cc.....	40	35	4*
Distilled water, 30 cc.....	68	43	7

* Only one root was well developed.

VII. Experiment with NaNO₃ and NaCl.

The antagonistic action of NO₃' and Cl' ions on each other was examined with the solution of sodium nitrate and chloride in the same manner as in the case of Experiment I. The transplanting of young rice seedlings, about 25 mm. high, took place on February 20 (1913) and the plants were measured on March 5 with the following result:

SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ NaNO ₃ , 30 cc.....	43	20	1
$\frac{N}{10}$ NaNO ₃ , 25 cc. + $\frac{N}{10}$ NaCl, 5 cc.....	63	35	7*
$\frac{N}{10}$ NaNO ₃ , 20 cc. + $\frac{N}{10}$ NaCl, 10 cc.....	59	30	6*
$\frac{N}{10}$ NaNO ₃ , 15 cc. + $\frac{N}{10}$ NaCl, 15 cc.....	55	35	6*
$\frac{N}{10}$ NaNO ₃ , 10 cc. + $\frac{N}{10}$ NaCl, 20 cc.....	50	25	5*
$\frac{N}{10}$ NaNO ₃ , 5 cc. + $\frac{N}{10}$ NaCl, 25 cc.....	57	30	6*
$\frac{N}{10}$ NaCl, 30 cc.....	46	25	3*
Distilled water, 30 cc.....	78	58	7

* Only one root was well developed.

VIII. Experiment with KNO₃ and KCl.

An experiment similar to the preceding one was made with potassium salts. The result obtained was as follows:

SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ KNO ₃ , 30 cc.....	45	25	3*
$\frac{N}{10}$ KNO ₃ , 25 cc. + $\frac{N}{10}$ KCl, 5 cc.....	60	35	6*
$\frac{N}{10}$ KNO ₃ , 20 cc. + $\frac{N}{10}$ KCl, 10 cc.....	50	35	6*
$\frac{N}{10}$ KNO ₃ , 15 cc. + $\frac{N}{10}$ KCl, 15 cc.....	48	30	6*
$\frac{N}{10}$ KNO ₃ , 10 cc. + $\frac{N}{10}$ KCl, 20 cc.....	50	40	7*
$\frac{N}{10}$ KNO ₃ , 5 cc. + $\frac{N}{10}$ KCl, 25 cc.....	72	40	7*
$\frac{N}{10}$ KCl, 30 cc.....	50	25	7*
Distilled water, 30 cc.....	78	58	7

* Only one root was well developed.

IX. Experiment with K₂SO₄ and NaCl.

The antagonism between K⁺, Na⁺, SO₄^{''} and Cl['] ions on each other was established with potassium sulphate and sodium chloride according to a method similar to that of Experiment VII. The following result was obtained:

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SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ K ₂ SO ₄ , 30 cc.....	47	35	7*
$\frac{N}{10}$ K ₂ SO ₄ , 25 cc. + $\frac{N}{10}$ NaCl, 5 cc.....	68	35	7*
$\frac{N}{10}$ K ₂ SO ₄ , 20 cc. + $\frac{N}{10}$ NaCl, 10 cc.....	59	35	6*
$\frac{N}{10}$ K ₂ SO ₄ , 15 cc. + $\frac{N}{10}$ NaCl, 15 cc.....	52	30	7*
$\frac{N}{10}$ K ₂ SO ₄ , 10 cc. + $\frac{N}{10}$ NaCl, 20 cc.....	60	33	7*
$\frac{N}{10}$ K ₂ SO ₄ , 5 cc. + $\frac{N}{10}$ NaCl, 25 cc.....	65	35	6*
$\frac{N}{10}$ NaCl, 30 cc.....	46	25	3*
Distilled water, 30 cc.....	78	58	7

* Only one root was well developed.

X. Experiment with Na₂SO₄ and KCl.

A similar experiment to the preceding one was made with sodium sulphate and potassium chloride. The following result which coincides with that of the previous experiment, was obtained:

SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ Na ₂ SO ₄ , 30 cc.....	45	20	2*
$\frac{N}{10}$ Na ₂ SO ₄ , 25 cc. + $\frac{N}{10}$ KCl, 5 cc.....	72	25	7*
$\frac{N}{10}$ Na ₂ SO ₄ , 20 cc. + $\frac{N}{10}$ KCl, 10 cc.....	65	26	7*
$\frac{N}{10}$ Na ₂ SO ₄ , 15 cc. + $\frac{N}{10}$ KCl, 15 cc.....	60	18	6*
$\frac{N}{10}$ Na ₂ SO ₄ , 10 cc. + $\frac{N}{10}$ KCl, 20 cc.....	64	25	7*
$\frac{N}{10}$ Na ₂ SO ₄ , 5 cc. + $\frac{N}{10}$ KCl, 25 cc.....	71	28	5*
$\frac{N}{10}$ KCl, 30 cc.....	50	25	7*
Distilled water, 30 cc.....	78	58	7

* Only one root was well developed.

XI. Experiment with Na₂SO₄ and KNO₃.

The antagonistic action of Na⁺, K⁺, SO₄^{''} and NO₃['] ions on each other was examined with sodium sulphate and potassium nitrate in the same manner as in Experiment VII. The result obtained was as follows:

SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ Na_2SO_4 , 30 cc.....	45	20	2*
$\frac{N}{10}$ Na_2SO_4 , 25 cc. + $\frac{N}{10}$ KNO_3 , 5 cc.....	65	35	6*
$\frac{N}{10}$ Na_2SO_4 , 20 cc. + $\frac{N}{10}$ KNO_3 , 10 cc.....	70	30	7*
$\frac{N}{10}$ Na_2SO_4 , 15 cc. + $\frac{N}{10}$ KNO_3 , 15 cc.....	60	30	3*
$\frac{N}{10}$ Na_2SO_4 , 10 cc. + $\frac{N}{10}$ KNO_3 , 20 cc.....	77	30	7*
$\frac{N}{10}$ Na_2SO_4 , 5 cc. + $\frac{N}{10}$ KNO_3 , 25 cc.....	60	35	4*
$\frac{N}{10}$ KNO_3 , 30 cc.....	45	25	3*
Distilled water, 30 cc.....	78	58	7

* Only one root was well developed.

XII. Experiment with K_2SO_4 and NaNO_3 .

The same antagonism as in the preceding experiment was again observed with potassium sulphate and sodium nitrate. A result similar to that of Experiment XI was obtained as will be seen in the following table:

SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ K_2SO_4 , 30 cc.....	47	35	7*
$\frac{N}{10}$ K_2SO_4 , 25 cc. + $\frac{N}{10}$ NaNO_3 , 5 cc.....	73	30	7*
$\frac{N}{10}$ K_2SO_4 , 20 cc. + $\frac{N}{10}$ NaNO_3 , 10 cc.....	60	20	5*
$\frac{N}{10}$ K_2SO_4 , 15 cc. + $\frac{N}{10}$ NaNO_3 , 15 cc.....	60	25	5*
$\frac{N}{10}$ K_2SO_4 , 10 cc. + $\frac{N}{10}$ NaNO_3 , 20 cc.....	60	35	6*
$\frac{N}{10}$ K_2SO_4 , 5 cc. + $\frac{N}{10}$ NaNO_3 , 25 cc.....	72	40	5*
$\frac{N}{10}$ NaNO_3 , 30 cc.....	43	20	1
Distilled water, 30 cc.....	78	58	7

* Only one root was well developed.

XIII. Experiment with KNO_3 and NaCl .

The antagonism between K^+ , Na^+ , NO_3^- and Cl^- ions in combination with each other was examined with potassium nitrate and sodium chloride in a manner similar to that of Experiment VII. The following result was obtained:

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SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ KNO ₃ , 30 cc.....	45	25	3*
$\frac{N}{10}$ KNO ₃ , 25 cc. + $\frac{N}{10}$ NaCl, 5 cc.....	65	30	5*
$\frac{N}{10}$ KNO ₃ , 20 cc. + $\frac{N}{10}$ NaCl, 10 cc.....	62	30	5*
$\frac{N}{10}$ KNO ₃ , 15 cc. + $\frac{N}{10}$ NaCl, 15 cc.....	60	25	4*
$\frac{N}{10}$ KNO ₃ , 10 cc. + $\frac{N}{10}$ NaCl, 20 cc.....	63	25	5*
$\frac{N}{10}$ KNO ₃ , 5 cc. + $\frac{N}{10}$ NaCl, 25 cc.....	75	35	7*
$\frac{N}{10}$ NaCl, 30 cc.....	46	25	1
Distilled water, 30 cc.....	78	58	7

* Only one root was well developed.

XIV. Experiment with NaNO₃ and KCl.

The antagonistic phenomenon observed in Experiment XIII was again tested with sodium nitrate and potassium chloride. The following result, which almost coincides with that of the preceding experiment, was obtained:

SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ NaNO ₃ , 30 cc.....	43	20	1
$\frac{N}{10}$ NaNO ₃ , 25 cc. + $\frac{N}{10}$ KCl, 5 cc.....	75	30	7*
$\frac{N}{10}$ NaNO ₃ , 20 cc. + $\frac{N}{10}$ KCl, 10 cc.....	65	35	6*
$\frac{N}{10}$ NaNO ₃ , 15 cc. + $\frac{N}{10}$ KCl, 15 cc.....	62	35	6*
$\frac{N}{10}$ NaNO ₃ , 10 cc. + $\frac{N}{10}$ KCl, 20 cc.....	64	25	4*
$\frac{N}{10}$ NaNO ₃ , 5 cc. + $\frac{N}{10}$ KCl, 25 cc.....	70	25	7*
$\frac{N}{10}$ KCl, 30 cc.....	50	25	7*
Distilled water, 30 cc.....	78	58	7

* Only one root was well developed.

Conclusions.

From the above results we may conclude as follows:

1. Sodium and potassium salts are antagonized by each other. The curve of antagonism between these salts shows two maxima and the location of these maxima is almost constant, occurring at the point of the proportion of 5 : 25. This coincides with the result which was observed by Osterhout¹⁵ on wheat seedlings.

2. The antagonism between these salts is due to cations as well as anions.

¹⁵ *Bol. Gaz.*, xlviii, pp. 98-104, 1900.

3. The antagonism between anions is small in comparison with that between cations.

PART IV. ON THE ANTAGONISM BETWEEN POTASSIUM AND MAGNESIUM OR CALCIUM IONS.

The antagonism between potassium and magnesium or calcium ions is especially interesting as shown in the experiment of Osterhout¹⁶ on wheat seedlings and of Loeb¹⁷ on *Fundulus*. In these experiments, it was shown that the toxicity of potassium ion is antagonized by magnesium or calcium ions, though calcium shows a more marked antagonism than magnesium. We have undertaken to investigate this relation in the case of growing rice seedlings, and accordingly we have made the following experiments:

I. *Experiment with KCl and MgCl₂.*

The antagonism between potassium and magnesium ions was established with the young seedlings of rice, about 25 mm. high, which were grown in distilled water from seeds of almost uniform size and specific gravity (1.185–1.200). Beakers of about 5.5 cm. in diameter and 7 cm. deep, each containing 30 cc. of the culture fluids, were used for the experiment. The seedlings were placed in the solutions on March 7, 1913. Five seedlings were grown in each culture in the greenhouse and the evaporated water was supplemented with distilled water from time to time so as to keep the solutions at their initial dilutions. On March 24, the difference in development in the respective cultures was very striking, and the following determinations were made:

SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ KCl, 30 cc.....	40	17	3
$\frac{N}{10}$ KCl, 25 cc. + $\frac{N}{10}$ MgCl ₂ , 5 cc.....	60	16	4
$\frac{N}{10}$ KCl, 20 cc. + $\frac{N}{10}$ MgCl ₂ , 10 cc.....	58	15	6
$\frac{N}{10}$ KCl, 15 cc. + $\frac{N}{10}$ MgCl ₂ , 15 cc.....	55	15	7
$\frac{N}{10}$ KCl, 10 cc. + $\frac{N}{10}$ MgCl ₂ , 20 cc.....	55	15	5
$\frac{N}{10}$ KCl, 5 cc. + $\frac{N}{10}$ MgCl ₂ , 25 cc.....	56	21	4
$\frac{N}{10}$ MgCl ₂ , 30 cc.....	43	23	3
Distilled water, 30 cc.....	63	47	11

¹⁶ *Bot. Gaz.*, xlv, p. 117, 1908; xlviii, pp. 98–104, 1909.

¹⁷ *Amer. Journ. of Physiol.*, iii, p. 327, 1900.

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In pure magnesium chloride solution the seedlings had grown only 18 mm. in eighteen days; in potassium chloride solution, only 15 mm.; while in distilled water the length of leaf had attained to 63 mm. Therefore, it is evident that potassium chloride and magnesium chloride have a poisonous action upon the growth of rice seedlings.

This poisonous effect largely disappears when we mix the two salts ($\text{MgCl}_2 + \text{KCl}$) in proper proportions. In the mixture $\frac{N}{10}$ KCl, 25 cc. + $\frac{N}{10}$ MgCl_2 , 5 cc., the growth of the seedlings was most vigorous and their height had reached to 60 mm. Therefore, it is evident that in the mixture of magnesium and potassium chloride in favorable proportion, the seedlings grow about twice as much as in pure solutions.

It will be noticed that decreasing the proportion of potassium or increasing the amount of magnesium beyond the optimum proportion causes unfavorable conditions for the growth of the seedlings. Accordingly, it is inferred that a small amount of magnesium retards the toxic effect of potassium, and on the other hand, potassium retards the injurious action of magnesium in large amount.

II. Experiment with KCl and CaCl_2 .

The antagonistic action of potassium and calcium ions on each other was examined with potassium and calcium chloride in the same manner as in the first experiment. The result obtained was as follows:

SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ KCl, 30 cc.....	40	17	3
$\frac{N}{10}$ KCl, 25 cc. + $\frac{N}{10}$ CaCl_2 , 5 cc.....	68	51	10
$\frac{N}{10}$ KCl, 20 cc. + $\frac{N}{10}$ CaCl_2 , 10 cc.....	65	25	8
$\frac{N}{10}$ KCl, 15 cc. + $\frac{N}{10}$ CaCl_2 , 15 cc.....	65	25	8
$\frac{N}{10}$ KCl, 10 cc. + $\frac{N}{10}$ CaCl_2 , 20 cc.....	65	25	8
$\frac{N}{10}$ KCl, 5 cc. + $\frac{N}{10}$ CaCl_2 , 25 cc.....	64	20	8
$\frac{N}{10}$ CaCl_2 , 30 cc.....	35	18	3
Distilled water, 30 cc.....	63	47	11

The result obtained was similar to that of the previous experiment, but it is clear that calcium has a more marked antagonistic action than magnesium and decidedly prevents the toxicity of the potassium ion.

Conclusion.

Potassium and magnesium or calcium salts are poisonous to the rice plant when used separately but when mixed together in suitable proportion the poisonous effect more or less completely disappears. The results coincide with those of Osterhout and form an important factor in the question of soil fertility.

PART V. CAN BARIUM AND STRONTIUM REPLACE THE ANTAGONISTIC ACTION OF CALCIUM?

It has been pointed out that the injurious action of certain metallic ions upon the growth of rice seedlings may be perfectly neutralized by the presence of calcium ions. It was of interest to experiment with barium and strontium, which are similar to calcium in chemical properties, to determine whether they exert an action similar to that of calcium. In order to investigate this problem we have used sodium and magnesium chloride as the toxic salts for the following experiments.

I. *Experiment with $MgCl_2$.*

Twenty beakers of about 5.5 cm. diameter and 7 cm. deep, served for the experiment. While one beaker which contained 30 cc. of distilled water served as check, the other nineteen beakers received the solutions noted in the table. Five seedlings, about 25 mm. high, which were grown in distilled water from seeds of almost uniform size and specific gravity (1.185–1.200), were transplanted in each of the respective beakers on February 25, 1913, and kept in the greenhouse. The evaporated water was supplemented with distilled water from time to time so as to keep the culture solutions at their initial concentration. On March 14, the difference in development in the respective cultures was very striking, and measurements were then made with the following result:

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SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
Distilled water, 30 cc.....	68	60	8
$\frac{N}{10}$ MgCl ₂ , 30 cc.....	53	12	1
$\frac{N}{10}$ MgCl ₂ , 25 cc. + $\frac{N}{10}$ CaCl ₂ , 5 cc.....	68	50	9
$\frac{N}{10}$ MgCl ₂ , 20 cc. + $\frac{N}{10}$ CaCl ₂ , 10 cc.....	65	40	10
$\frac{N}{10}$ MgCl ₂ , 15 cc. + $\frac{N}{10}$ CaCl ₂ , 15 cc.....	62	35	8
$\frac{N}{10}$ MgCl ₂ , 10 cc. + $\frac{N}{10}$ CaCl ₂ , 20 cc.....	60	20	8
$\frac{N}{10}$ MgCl ₂ , 5 cc. + $\frac{N}{10}$ CaCl ₂ , 25 cc.....	52	15	6
$\frac{N}{10}$ CaCl ₂ , 30 cc.....	44	12	8
$\frac{N}{10}$ MgCl ₂ , 25 cc. + $\frac{N}{10}$ BaCl ₂ , 5 cc.....	40	12	1
$\frac{N}{10}$ MgCl ₂ , 20 cc. + $\frac{N}{10}$ BaCl ₂ , 10 cc.....	33	10	1
$\frac{N}{10}$ MgCl ₂ , 15 cc. + $\frac{N}{10}$ BaCl ₂ , 15 cc.....	28	8	1
$\frac{N}{10}$ MgCl ₂ , 10 cc. + $\frac{N}{10}$ BaCl ₂ , 20 cc.....	28	7	1
$\frac{N}{10}$ MgCl ₂ , 5 cc. + $\frac{N}{10}$ BaCl ₂ , 25 cc.....	28	5	1
$\frac{N}{10}$ BaCl ₂ , 30 cc.....	24	8	1
$\frac{N}{10}$ MgCl ₂ , 25 cc. + $\frac{N}{10}$ SrCl ₂ , 5 cc.....	60	12	3
$\frac{N}{10}$ MgCl ₂ , 20 cc. + $\frac{N}{10}$ SrCl ₂ , 10 cc.....	45	8	3
$\frac{N}{10}$ MgCl ₂ , 15 cc. + $\frac{N}{10}$ SrCl ₂ , 15 cc.....	40	5	2
$\frac{N}{10}$ MgCl ₂ , 10 cc. + $\frac{N}{10}$ SrCl ₂ , 20 cc.....	35	7	1
$\frac{N}{10}$ MgCl ₂ , 5 cc. + $\frac{N}{10}$ SrCl ₂ , 25 cc.....	30	9	1
$\frac{N}{10}$ SrCl ₂ , 30 cc.....	22	8	1

The result shows that the presence of calcium in proper proportion can exert only a beneficial action, while in the case of barium, on the contrary, a depression resulted. Although strontium in suitable proportion retarded the toxic action of magnesium, it is far inferior to calcium.

II. *Experiment with NaCl.*

Twenty beakers, each containing 30 cc. of culture fluids, served for the experiment. The culture solutions were applied in the same proportion as in Experiment I using NaCl instead of MgCl₂.

Five seedlings, about 20 mm. high, were transplanted on March 7, 1913, and kept in the greenhouse. The evaporated water was supplemented with distilled water from time to time. The plants had developed very well with remarkable differences in growth. The plants were measured on March 24 with the following result which coincides with that of the preceding experiment.

SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
Distilled water, 30 cc.....	65	50	9
$\frac{N}{10}$ NaCl, 30 cc.....	44	13	1
$\frac{N}{10}$ NaCl, 25 cc. + $\frac{N}{10}$ CaCl ₂ , 5 cc.....	70	40	9
$\frac{N}{10}$ NaCl, 20 cc. + $\frac{N}{10}$ CaCl ₂ , 10 cc.....	70	40	9
$\frac{N}{10}$ NaCl, 15 cc. + $\frac{N}{10}$ CaCl ₂ , 15 cc.....	60	25	8
$\frac{N}{10}$ NaCl, 10 cc. + $\frac{N}{10}$ CaCl ₂ , 20 cc.....	56	20	8
$\frac{N}{10}$ NaCl, 5 cc. + $\frac{N}{10}$ CaCl ₂ , 25 cc.....	50	20	6
$\frac{N}{10}$ CaCl ₂ , 30 cc.....	44	22	6
$\frac{N}{10}$ NaCl, 25 cc. + $\frac{N}{10}$ BaCl ₂ , 5 cc.....	40	20	3
$\frac{N}{10}$ NaCl, 20 cc. + $\frac{N}{10}$ BaCl ₂ , 10 cc.....	41	18	5
$\frac{N}{10}$ NaCl, 15 cc. + $\frac{N}{10}$ BaCl ₂ , 15 cc.....	30	15	3
$\frac{N}{10}$ NaCl, 10 cc. + $\frac{N}{10}$ BaCl ₂ , 20 cc.....	30	20	3
$\frac{N}{10}$ NaCl, 5 cc. + $\frac{N}{10}$ BaCl ₂ , 25 cc.....	28	17	3
$\frac{N}{10}$ BaCl ₂ , 30 cc.....	29	10	3
$\frac{N}{10}$ NaCl, 25 cc. + $\frac{N}{10}$ SrCl ₂ , 5 cc.....	50	22	6
$\frac{N}{10}$ NaCl, 20 cc. + $\frac{N}{10}$ SrCl ₂ , 10 cc.....	47	20	4
$\frac{N}{10}$ NaCl, 15 cc. + $\frac{N}{10}$ SrCl ₂ , 15 cc.....	45	16	5
$\frac{N}{10}$ NaCl, 10 cc. + $\frac{N}{10}$ SrCl ₂ , 20 cc.....	40	10	4
$\frac{N}{10}$ NaCl, 5 cc. + $\frac{N}{10}$ SrCl ₂ , 25 cc.....	36	16	4
$\frac{N}{10}$ SrCl ₂ , 30 cc.....	28	15	3

Conclusion.

The injurious effect of certain metallic ions upon the growth of rice seedlings may be perfectly counteracted only by the presence of calcium ions. Strontium ions can exert an influence only slightly retarding the toxicity of the metallic ions. Barium ion not only has no beneficial action, but a depressing effect is observed. Consequently, it is concluded that barium and strontium cannot replace the antagonistic action of calcium.

THE DETERMINATION OF OXYBUTYRIC ACID.¹

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In 1908 one of us described a method² for the determination of oxybutyric acid in urine, based upon its oxidation by chromic acid with the formation of acetone, the latter being determined by standard iodine and thiosulphate solutions in the usual way. The procedure was combined with the determination of acetone, preformed and from diacetic acid, so that the technique as described accomplished the determination of the three "acetone bodies" in the same sample of urine and with practically the same reagents.

The advantages of this method for the determination of oxybutyric acid over those based upon the optical activity of the extracted acid are believed to be the shorter time required; the fact that even very small as well as large amounts may be determined with the same degree of accuracy; and the combined determination of the related "acetone bodies" in the same sample of urine in one procedure.

At the time this method was described it was believed that practically theoretical results were obtained when the specified conditions were adhered to. This belief was based on a series of determinations on a solution of the inactive acid which had been purified by recrystallization of the sodium salt, the titrated acidity being taken as the criterion of the concentration. These results varied

¹ A part of the work described in this paper was done during the summers of 1911 and 1912 in the Biochemical Laboratory of the Harvard Medical School. My thanks are due Professor Folin for placing the facilities of his laboratory at my disposal. I am indebted also to Professor Christian and to Dr. Joslin of Harvard, to Dr. I. Greenwald of the Chemical Laboratory of the Montefiore Home in New York, and to Doctor Jesse Myer of Washington University, for a supply of diabetic urine.—P. A. S.

² Shaffer: This *Journal*, v, p. 211, 1908.

from 98.8 to 103.3 per cent of the theoretical values, and were accepted as showing that the oxidation and formation of acetone is practically a quantitative reaction. None of the known salts of oxybutyric acid proved suitable to use as a standard, and the levic acid was not used for the purpose because of an inability to get it to crystallize and of a lack of confidence at the time in its published specific rotation.

Gorslin and Cooke³ have suggested slight modifications in the method but did not question its accuracy. Mondschcin⁴ also has published figures indicating that he obtained satisfactory results. It now appears, however, that the claim of theoretical results for the method is not altogether correct.

Embden and Schmitz,⁵ in criticizing the method, state that the results are probably somewhat too low since they obtained only a fraction of the optical value when the raw extracts prepared from urines for the polarization method were oxidized by bichromate. With urines containing much of the acid 80 to 90 per cent or more of the optical values were obtained on oxidizing the extracts, while the extracts from urines containing little of the acid gave on oxidation only a small part of the optical value. Embden and Schmitz accordingly believe that the ether extracts of such urines contain levo-rotary substances other than the oxybutyric acid and that the optical values are therefore too high, while the results of the oxidation method are somewhat too low. They suggest that the true results lie between the values obtained by the polariscopic and oxidation methods.

These findings together with our intention of using the method for other work involving the determination of oxybutyric acid and acetone in blood and tissues, for which there existed no suitable procedure, led us to undertake a review of the various optical points of the oxidation method and of the method based upon the activity of the ether extracts.

A series of determinations by the oxidation method on solutions of β -oxybutyric acid as extracted from the urine by ether (Black's method) gave results which confirmed the statement of Embden

³ Gorslin and Cooke: *this Journal*, x, p. 291, 1911-12.

⁴ Mondschcin: *Biochem. Zeitschr.*, xlii, p. 95, 1912.

⁵ Embden and Schmitz: *Handbuch der biochemischen Arbeitsmethoden*, 1910, Bd. iii, p. 934.

and Schmitz and show that the optical values of such extracts are greater than those obtained by oxidation.

SOLUTION	OPTICAL VALUE	OXIDATION METHOD	PER CENT OF OPTICAL VALUE
	grams in 100 cc.		
1. From diabetic urine..... ($\alpha = -14.84^\circ$, $l = 2.2$ [α] _D ²⁰ = -24.12°)	28.0	26.4 25.75 26.1 25.95 25.67 25.83 25.33 25.86	94.5 92.0 93.3 92.7 91.8 92.5 90.5 92.4
Average.....			
2. From diabetic urine..... ($\alpha = 1.92^\circ$, $l = 2.$)	3.98	3.40 3.42	85.5 86.0
3. From non-diabetic urine..... ($\alpha = -0.76^\circ$, $l = 2.$)	1.576	1.265	80.3
4. From non-diabetic urine..... ($\alpha = -0.83^\circ$, $l = 2.$)	1.72	1.44	84.0
5. From non-diabetic urine..... ($\alpha = -1.69^\circ$, $l = 2.$)	3.50	2.97 3.07 3.05	85.0 87.6 87.2
6. From diabetic urine.....	2.06	2.05	98.6
7. From diabetic urine.....	2.10	2.05	97.8
8. From diabetic urine.....	2.04	1.95	95.5
9. From diabetic urine.....	2.06	1.92	93.3
10. From normal urine ⁶	0.58	0.21	48.0

According to these results the values obtained on oxidation of the urine extracts vary from about 48 per cent of the optical value in the case of the extract from normal urine (the greater part of which was not oxybutyric acid) to about 98 per cent, usually between 85 and 95 per cent. These findings led to a somewhat

⁶ The same solution after precipitation by basic lead acetate and ammonia gave by the oxidation method values equivalent to 0.058 per cent β -oxybutyric acid, or only 10 per cent of the original optical value; 90 per cent of the levo-rotary substance extracted from this urine was therefore not oxybutyric acid. Oxybutyric acid is not precipitated when its solutions are treated with basic lead acetate and ammonia. Occasionally it may appear that there is a loss of 1 to 2 per cent, but such amounts are within the limits of accuracy of which the method is ordinarily capable, and are negligible. The following example shows the difference sometimes observed: 0.5885 gram of pure calcium zinc oxybutyrate was dissolved and made up

lengthy study of the factors which might explain the discrepancies. In order first to test whether the oxidation method as carried out actually yields low results, it was necessary to carry out a series of determinations, on solutions of synthetic and *l*-oxybutyric acid of known purity. Since the methods of preparation and purification differ in some particulars from those heretofore used, the details are given below.

Purification of β -oxybutyric acid.

The impure *l*-acid as extracted from urine, especially after fermentation of the sugar, invariably is contaminated with other organic acids. For the removal of these Magnus-Levy⁷ has suggested the neutralization of the extract with calcium carbonate and the addition of an equal volume of alcohol to the solution of calcium salts, under which conditions he claims the salts of most of the other acids separate, the calcium oxybutyrate remaining in solution. This is not in accordance with our experience; as a rule one gets no precipitation. Calcium lactate and the salts of whatever other acids that may be present are quite soluble in 50 per cent or even much stronger alcohol. If, however, instead of calcium the acids are converted into the zinc salts and alcohol added, the lactate and perhaps also other salts are fairly completely precipitated on standing, while the zinc oxybutyrate, quite contrary to statements in the literature, is very soluble in both alcohol and water, and remains in solution. We have used this procedure. After filtering off the precipitated zinc salts the alcohol is boiled off, the

to 250 cc. 100-cc. portions of this solution were (1) treated with basic lead acetate and ammonia, diluted to 250 cc. and filtered; and (2) diluted to 250 cc. and filtered. Determinations were made by the oxidation method in 75-cc. portions of each filtrate, equivalent to 30 cc. of the original solution which contained 0.0706 gram salt or 0.0567 gram oxybutyric acid.

	Mgm. acetone found	Oxybutyric acid	Per cent of theory (wt.)
Without lead.....	29.1	52.2	92.0
	29.2	52.4	92.4
	29.0	52.0	91.8
After lead precipitation	28.4	51.0	90.0
	28.3	50.8	89.5
	28.6	51.3	90.5

⁷ Magnus-Levy: *Ergeb. d. inn. Med. u. Kinderheilk.*, i, p. 414, 1908.

syrup cooled, strongly acidified with sulphuric acid (50 per cent) and plaster or anhydrous sodium sulphate added and the mixture allowed to harden. If the syrup is much colored, pure bone black may be added before the plaster. The coarsely powdered material is then extracted with ether in a Soxhlet apparatus. After removing the ether from the extract the residue is dissolved in 10 parts or less of water and if necessary shaken cold with a little pure bone black and filtered.

The inactive acid is conveniently made according to Wislicenus⁸ by the reduction of aceto-acetic ester by sodium amalgam. The free acid is best isolated by extraction with ether after evaporating, acidifying and dehydrating the solution of the salt with plaster or sodium sulphate.

For the further purification of the acid, the salts hitherto used, with the exception of the sodium salt which is very deliquescent, have in our hands not proved suitable. Repeated and varied efforts to get the *l*-acid to crystallize have so far not been successful. A new double salt of calcium and zinc was, however, discovered which has been of considerable service. This salt, which is quite stable, crystallizes in long needles or needle-like plates, and while soluble in about 10 parts of water (*dl*-salt in 7 parts) is much less soluble than the other salts of the acid with which we have worked. The salt is prepared as follows:

Calcium-zinc oxybutyrate. Equal parts of the free acid are neutralized by warming with zinc carbonate and calcium carbonate respectively, the solutions filtered and poured together. Both the calcium and the zinc salts are very soluble, but when mixed, the double salt crystallizes almost at once if the concentration is greater than 10 per cent (14 per cent in the case of the *dl*-salt). The greater part of that remaining in solution is precipitated beautifully crystalline after a few hours on adding an equal volume of hot alcohol. It may be repeatedly crystallized by precipitation by alcohol, though the final crystallization should be from water by evaporation, because the alcohol causes a slight hydrolysis and the precipitation also of a little zinc hydroxide which remains undissolved when these preparations are again dissolved in water. After recrystallizing several times from water, the preparations are practically pure. The free acid may be recovered by acidify-

⁸ Wislicenus: *Ann. d. Chem.*, cxlix, p. 205, 1869.

ing the solutions of the salt, setting with sodium sulphate⁹ and extraction with ether.

Determinations of the specific rotation of the recrystallized *l*-calcium zinc β -oxybutyrate in 3 per cent to 9 per cent solutions gave the average value: $[\alpha]_D^{20} = -16.26^\circ$

PREPARATION I (a). Five times recrystallized from alcohol and once from water.

0.8742 gram in 10 cc. $l=2.2$, $\alpha = -3.12^\circ$

$$[\alpha]_D^{20} = -16.25^\circ$$

PREPARATION I (b). After again recrystallizing from water.

2.9646 gram in 50 cc. $l=2.2$, $\alpha = -2.10^\circ$

$$[\alpha]_D^{20} = -16.15^\circ$$

PREPARATION II (a). Recrystallized once from alcohol and twice from water.

3.9630 grams in 50 cc. $l=2.2$, $\alpha = -2.84^\circ$

$$[\alpha]_D^{20} = -16.28^\circ$$

PREPARATION II (b). After again recrystallizing from water.

3.7584 grams in 50 cc. $l=2.2$, $\alpha = -2.69^\circ$

$$[\alpha]_D^{20} = -16.26^\circ$$

Analysis of this salt for ash ($\text{CaO} + \text{ZnO}$) and calcium corresponds to the formula $\text{CaZn}(\text{C}_4\text{H}_7\text{O}_3)_4$.

Ash: Cautiously ignited to constant weight with small portions of pure ammonium nitrate.

I. 1.2279 grams substance = 0.3243 gram ash = 26.41 per cent.

II. 0.5194 gram substance = 0.1386 gram ash = 26.68 per cent.

III. 0.7163 gram substance = 0.1898 gram ash = 26.50 per cent.

Average found = 26.53 per cent. Theory = 26.55 per cent.

Calcium determination by precipitation as oxalate after removal of zinc by hydrogen sulphide gave the following results:

I. 1.3014 grams salt = 0.3661 gram calcium oxalate ($\text{Ca}(\text{COO})_2 + \text{H}_2\text{O}$)
= 7.77 per cent calcium.

II. 1.3550 grams salt = 0.3837 gram calcium oxalate
= 7.76 per cent calcium.

III. 1.2817 grams salt = 0.3648 gram calcium oxalate
= 7.79 per cent calcium.

Theory = 7.74 per cent.

⁹ Powdered anhydrous sodium sulphate frequently contains small amounts of material soluble in ether which appear in the extracts. The substance may be removed by several recrystallizations of sodium sulphate from water.

The salt melts with decomposition and not sharply at about 240°C.

Determinations by the oxidation method on solutions of the above preparations of *l*-double salt and on solutions of the free acid obtained therefrom gave the following results.

For the determinations the contents of the distilling flask containing the oxybutyric acid was diluted to about 600 cc., 30 cc. of sulphuric acid (sp. gr. 1.59) added, and a total of about 0.5 gram of $K_2Cr_2O_7$ in very dilute solution dropped in during the distillation which was continued about three and one-half hours. The acetone in the distillates was titrated with iodine and thiosulphate solutions.

I (a). 25 cc. of a solution containing 0.0874 gram salt = 0.0703 gram acid were taken for each determination.

Grams acetone found	Grams oxybutyric acid	Per cent of theory
0.0358	0.0642	91.4
0.0364	0.0653	93.0
0.0356	0.0639	90.9
0.0356	0.0639	90.9

I (b). 50 cc. of a solution containing 0.0886 gram double salt = 0.0712 gram acid were used for each determination, which was carried out as above.

Grams acetone found	Grams oxybutyric acid	Per cent of theory
0.0372	0.0668	93.8
0.0373	0.0670	94.1
0.0371	0.0665	93.4

I (c). Preparation I (b) again recrystallized from water. 0.0885 gram salt = 0.0711 gram acid was taken for each determination and carried out as above.

Grams acetone found	Grams oxybutyric acid	Per cent of theory
0.0373	0.0669	94.2
0.0370	0.0664	93.4

II (a). 0.0887 gram salt = 0.0713 gram acid taken for each determination.

Grams acetone found	Grams oxybutyric acid	Per cent of theory
0.0367	0.0658	92.5
0.0367	0.0658	92.5
0.0367	0.0658	92.5

II (b). 0.0884 gram salt = 0.0711 gram acid taken for each determination.

Grams acetone found	Grams oxybutyric acid	Per cent of theory
0.0367	0.0658	92.6
0.0367	0.0658	92.6
0.0368	0.0660	92.8
0.0366	0.0656	92.3

II (c). A solution of the *l*-acid isolated from preparation II (b).

OPTICAL VALUE: $([\alpha]_D^{20} = -24.12^\circ) = 4.45$ per cent.

Acetone found in 50 cc. of a dilution equivalent to 1 cc. of original solution containing 0.0445 gram acid (optical value):

Grams acetone found	Grams oxybutyric acid	Per cent of theory (optical value)
0.0232	0.0416	93.5
0.0230	0.0412	92.7
0.0228	0.0409	92.0

III. *l*-Double salt three times recrystallized from water. 25 cc. containing 0.0730 gram salt = 0.0586 gram acid was used for each determination carried out as above.

Grams acetone found	Grams oxybutyric acid	Per cent of theory (weight of salt)
0.0299	0.0536	91.5
0.0305	0.0547	93.3
0.0297	0.0533	91.0
0.0299	0.0536	91.5

IV. Synthetic *dl*-calcium zinc salt, recrystallized three times from water. 50 cc. solution containing 0.08912 gram salt = 0.0715 gram acid taken for each determination.

Grams acetone found	Grams oxybutyric acid	Per cent of theory
0.0371	0.0665	93.0
0.0370	0.0663	92.8
0.0375	0.0672	94.0

The results by the oxidation method upon the supposedly pure preparations point to the conclusion that the method as carried out yields values from 5 to 10 per cent less than the theory. We have made many efforts to find conditions which would give theoretical results, but so far without complete success. Other oxidizing agents have not proved suitable; raising the temperature by inert salts has proved futile; and the substitution of other acids for sulphuric, or changes in the concentration of acid, do not give higher results. The low results are probably to be explained by a portion of the aceto-acetic acid undergoing the well-known acid decomposition, with the formation of acetic acid instead of acetone. On boiling solutions of pure oxybutyric acid or its salts in 5 per cent sulphuric acid the distillates contain little or no acid, but if a large excess of bichromate is added, considerable amounts of acid other than carbonic (about 50 cc. $\frac{N}{16}$ in some experiments from 1 gram of calcium zinc salt) pass over, and under these conditions acetic acid is readily recognized in the distillate. It is therefore probable that small amounts of acetic acid are thus formed even

when the bichromate is added very slowly, and that even under the best conditions this decomposition amounts to about 5 per cent of the oxybutyric acid present.

It is not likely that there is a decomposition of acetone once it is formed, for experiments show that acetone is unaffected under the conditions and passes into the distillate very rapidly. Nor does the trouble lie in the titration of the acetone (see page 283).

Somewhat higher results can be obtained by adding the bichromate *very* slowly and continuing the distillation for a correspondingly long period; and conversely, as was pointed out in the first paper, if the bichromate is added too rapidly, very low results are found, and as noted above considerable amounts of acetic acid are formed. The following determinations were made as described on page 271, except that instead of 0.4 to 0.6 gram $K_2Cr_2O_7$, only 90 mgm. were added in very dilute solution during the first two and three-quarter hours of distillation and another 90 mgm. during a subsequent hour, the distillation being continued with slow boiling for four hours. The amount of bichromate added was still more than twice that theoretically required to oxidize the oxybutyric acid to aceto-acetic acid. The results are expressed as oxybutyric acid:

	CALCULATED FROM WT. OF SALT	AVERAGE OF THREE DETERMINATIONS AS USUALLY CARRIED OUT. 0.4-0.6 GM. $K_2Cr_2O_7$		BY SLOW OXIDATION. 0.18 GM. $K_2Cr_2O_7$	
		grams	per cent	grams	per cent
I.....	0.0711	0.0667	93.7	0.068	95.7
II.....	0.0713	0.0657	92.3	0.0696	97.5
III.....	0.0715	0.0667	93.5	0.0694	97.0
IV.....	0.0711			0.0676	95.2
V.....	0.0445	0.0412	92.7	0.0425	95.5

It is possible that still slower addition of bichromate and still longer distillation would yield nearly 100 per cent, but the accuracy of the method would not thereby be increased because when carried out as originally described, the results are practically constant, though it now appears that they represent only 90 per cent to 95 per cent of the true values. It seems preferable to retain the original directions and to add a correction of about 10 per cent to the result.

The oxidation method as applied to urine.

At the time of the first attempts to apply the oxidation of oxybutyric acid to its determination in urine it was found that several substances which normally or occasionally are present, interfere with the results by yielding products which use up iodine when the distillates are titrated. The substances considered were glucuronic acids, sugar, lactic, butyric and formic acids, perhaps leucine,¹⁰ phenols and some unidentified substances. The possibility of any material interference by these substances was effectively obviated by the introduction of three modifications; glucuronic acids and sugar are removed by preliminary precipitation with basic lead acetate and ammonia; phenol, butyric and formic acid if present are removed during the first distillation of the acidified filtrate, which also removes the acetone (preformed and from aceto-acetic acid), the latter being titrated after redistillation from alkali. Lactic acid if present is in part converted into acetic aldehyde on oxidation with bichromate, and to obviate possible interference from this source the distillate containing it and the acetone derived from the oxybutyric acid is redistilled from alkali and hydrogen peroxide, which completely holds back as acetate the small amounts of aldehyde which may have been formed. The effect of the unidentified substances is almost wholly removed by the precipitation with lead and the final redistillation with hydrogen peroxide.

The chief criticism of the method advanced by Embden and Schmitz¹¹ is that sugar, which of course is usually present in urines in which it is desired to determine oxybutyric acid, gives rise on oxidation with bichromate to substances which use iodine when the distillates are titrated and that, contrary to the finding of one of us, this interfering product is not removed on redistilling with alkali and hydrogen peroxide, and that therefore the method cannot be applied directly to urines containing sugar. We have confirmed this statement to the extent that glucose, when oxidized with sulphuric acid and bichromate, does give (as was pointed out

¹⁰ Later experiments have shown that leucine does not yield acetone when boiled with sulphuric acid and bichromate under the conditions of the method.

¹¹ *Loc. cit.*

in the first paper)¹² small amounts of a volatile substance which reacts with hypoiodate, and that it is frequently not wholly removed by alkali and hydrogen peroxide, which was first believed to be the case. The following experiment illustrates this behavior:

2 grams glucose in 600 cc. water plus 10 cc. concentrated H_2SO_4 were distilled, dropping in 3 per cent $\text{K}_2\text{Cr}_2\text{O}_7$. The distillate was redistilled after adding 30 cc. of 3 per cent H_2O_2 and 5 cc. of 10 per cent NaOH .

	cc. $\frac{N}{10}$ iodine used
2 grams glucose.....	5.2
Duplicate.....	4.3
1 gram glucose.....	0.6
Duplicate.....	2.8

These results as well as the criticism by Embden and Schmitz are, however, quite immaterial so far as the method is concerned because it has never been suggested that the method be performed on the urine direct, but only after precipitation by basic lead acetate and ammonia, which wholly removes the effect of the sugar as the following experiments show.

A series of determinations were carried out on a normal urine with and without the addition of 3 grams of glucose per 100 cc. urine. In each instance to 50 cc. urine were added 50 to 75 cc. basic lead acetate solution and 15 cc. concentrated ammonium hydroxide. The mixture was diluted to 500 cc. and 200 cc. of the filtrate, equivalent to 20 cc. urine, were diluted and distilled first with 30 cc. 1 to 1 sulphuric acid (distillate A which was discarded), and then with the gradual addition of potassium bichromate. Some of the distillates were titrated direct and others were redistilled after adding 5 cc. of 10 per cent NaOH and 20 cc. of 3 per cent H_2O_2 .

cc. $\frac{N}{10}$ iodine used.

NORMAL URINE NOT REDISTILLED	URINE PLUS 3 PER CENT SUGAR NOT REDISTILLED	URINE PLUS 3 PER CENT SUGAR REDISTILLED
0.4	0.5	0.6
0.6	0.6	0.8
0.6	0.6	0.7
0.6	0.7	

These figures are equivalent to about 30 mgm. of acetone from oxybutyric acid per liter of urine. It is probable that this repre-

¹² The presence of glucose is clearly without effect upon the results when the method is properly carried out. This *Journal*, v, p. 218, 1908.

sents actual oxybutyric acid present in normal urine, but whether or not this is so, the amount is negligible so far as it affects the results obtained from urines containing significant amounts of oxybutyric acid.

When a known amount of oxybutyric acid is added to normal urine, the results by the oxidation method correspond to about 90 per cent of the amount added, as illustrated by the following experiment.

2.540 grams pure *l*-calcium zinc salt were dissolved in 250 cc. normal urine (equivalent to 8.16 grams of the free acid per liter of urine).

	Found grams per liter	10 per cent added to result
Urine without oxybutyric acid.....	0.029	
Urine plus oxybutyric acid.....	7.54	8.29
	7.50	8.25
	7.40	8.14
	7.30	8.03
Average.....		8.18
Amount oxybutyric acid added		8.16

There are no essential changes in the procedure from the technique as originally described, but for convenience the description may be repeated here with the addition of some further details.

* The oxidation method, combined with the preliminary distillation for the removal and determination of acetone and diacetic acid, is carried out as follows: From 25 cc. to 100 cc. or more of urine (usually 50 cc.) are measured with a pipette into a 500 cc. volumetric flask containing 200 cc. to 300 cc. of water. Basic lead acetate solution (U. S. P.) is added in amount equal to the urine used¹³ and the liquid well mixed. Strong ammonia water, about half the volume of the lead acetate, is next poured in, the flask diluted to the mark with water, shaken, and after a few minutes' standing, the liquid is filtered, preferably through a folded filter, 200 cc. of the filtrate is measured into a round bottom flask (800 cc. or liter Kjeldahls are convenient) diluted with water to about 600 cc., 15 cc. of the concentrated sulphuric acid and tale or boiling stone added, and the mixture distilled until about 200 cc. of the distillate have collected (Distillate A).

The distilling flask must be fitted with a dropping tube and water run in from time to time to prevent the volume in the flask from becoming less than 400 to 500 cc.

¹³ If the urine contains but little or no sugar only half the amount or less of lead acetate should be used.

Distillate A, which contains the acetone preformed and from aceto-acetic acid, and which should be collected in a second Kjeldahl flask, is redistilled (for about twenty minutes) after adding 10 cc. of 10 per cent sodium hydroxide.¹⁴ The distillate so obtained (A_2) is titrated with standard iodine and thiosulphate solutions.

The residue of urine plus sulphuric acid from which Distillate A was obtained is again distilled¹⁵ dropping in either water, when necessary to keep the volume between 400 and 600 cc., or a dilute solution of potassium bichromate. From 0.5 gram to 1 gram of bichromate will usually be sufficient, and not more than 1 gram should be added unless the liquid turns green indicating a great reduction to chromium sulphate; very rarely 2 or 3 grams of bichromate may be necessary, especially if the sugar has not been completely removed.

A 10 per cent solution of potassium bichromate is kept on hand and 10 cc. of this, diluted to 100 cc. are measured out for each determination. 20 cc. of the dilute solution (0.2 gram $K_2Cr_2O_7$) are first added slowly through the dropping tube and then 10-cc. portions every fifteen or twenty minutes until the whole has been added. Should the liquid become markedly green the bichromate must be added at correspondingly shorter intervals and in amount sufficient to maintain a slight red-yellow color of the chromic acid, which may be detected even in the presence of the green. The distillation is continued with moderate boiling for from two to three hours. The distillate (B), which should be collected in a liter flask to avoid transference, is again distilled for about twenty minutes after adding 10 cc. of 10 per cent sodium hydroxide and 25 cc. of 3 per cent hydrogen peroxide. The flask must be heated cautiously until the peroxide has decomposed. This distillate (B_2) is titrated with the standard iodine and thiosulphate.

1 cc. of $\frac{N}{10}$ iodine = 0.968 mgm. acetone = 1.736 mgm. oxybutyric acid,
or

1 cc. of $\frac{1.035N}{10}$ iodine (= 13.13 mgm. I_2) = 1 mgm. acetone = 1.793 mgm.
oxybutyric acid.

Comparison with results by the extraction method.

The fact that the oxidation method gives results for oxybutyric acid which are uniformly from 5 to 10 per cent too low, explains in part the differences between the results by this method and the values calculated from the levo rotation of the ether extracts,

¹⁴ In many instances, when a high degree of accuracy is not required, this redistillation may be omitted and "distillate A" titrated direct; the results so obtained are slightly higher than those after redistillation from alkali.

¹⁵ The distillation is actually not interrupted; after "A" has collected, a new receiving flask is adjusted and bichromate solution slowly added through the dropping tube. The receiving tube of the condenser must dip below the surface of the water in the receiving flask.

but it appears that this does not explain the differences in all cases. Were this the only factor the results by the oxidation method would regularly be from 90 to 95 per cent of the optical values of the acid extracted by ether, whether the oxidation method were carried out on the urine as usual, or on the solution of the extracted acid. Occasionally this is the case as illustrated by the following figures:

Diabetic Urine. Grams oxybutyric acid per liter.

EXTRACTION METHOD (Black ¹⁶)	OXIDATION METHOD	
	On urine	On extracts
4.65	4.75	4.61
5.25	4.73	5.12
5.10	4.80	4.87
5.15	4.77	4.81

But frequently the results from oxidation of the extracts are much below the expected 90 to 95 per cent of their optical value; and after treating the extracts with basic lead acetate and ammonia the oxidation results are still lower. As examples the following may be cited; the results are expressed as grams of oxybutyric acid per liter:

Oxidation method on urine.....	7.71
	7.85
Black's method (3 hours' extraction).....	7.88
Oxidation of extract.....	6.32=80 per cent
Black's method (4 hours' extraction).....	8.60
Oxidation of extract.....	7.21=84 per cent
Black's method (4 hours' extraction).....	8.76
Oxidation of extract.....	7.58=86 per cent
Oxidation of extract after lead precipita- tion.....	7.10=81 per cent

The results from solutions 2, 3, 4, 5 and especially solution 10, from normal urine on page 267, show the same point differences

¹⁶ Black: this *Journal*, v, p. 209, 1908. For the extraction method 50 cc. to 200 cc. of urine were taken and Black's directions followed except that regular Soxhlet extractors were used. The extraction was continued for from six to ten hours, usually in two periods. After removing the ether by cautious warming on the water bath, the residue was dissolved to 25 cc., the solution was shaken cold with a little purified bone black, filtered and polarized. Parts of these solutions were then subjected to determination by the oxidation method, with or without a preliminary precipitation with basic lead acetate and ammonia.

between the results by oxidation and by polarization which are greater than the 5 or 10 per cent already accounted for. As first suggested by Embden and Schmitz¹⁷ there would appear to be present small amounts of a levo-rotary ether-soluble substance which tend to give somewhat too high results by the polariscopic methods. The identity of the substance we do not know. It may be precipitated by basic lead acetate and ammonia, and this preliminary treatment might well be adopted by those who use the extraction method.

There are two other points which we have encountered which tend to low results by Black's technique of the extraction method. Although Black's plan of dehydrating the evaporated urine with plaster and extraction of the dry material is far more rapid and convenient than the Magnus-Levy liquid extraction, the complete extraction frequently requires rather longer than the three or four hours recommended by Black. As a rule we have found in the extract from the second four-hour period 5 or 10 per cent of the amount obtained during the first four hours.

A more serious objection is the occasional apparent decomposition of a part of the oxybutyric acid during extraction. After ten hours and longer extraction we have repeatedly found only about 90 per cent of the acid which we had added to urine or other solutions. Thus from the urine mentioned on page 276, to 250 cc. of which were added 2.540 grams of pure *l*-calcium zinc oxybutyrate (=8.16 grams oxybutyric acid per liter), duplicate determinations on 100-cc. portions by the Black method (10 hours' extraction) gave 7.675 grams and 7.675 grams per liter.

$$(\alpha = -1.63^\circ, l = 2.2, [\alpha]_D^{20} = -24.12^\circ)$$

Subtracting the blank equivalent to the extract from the urine alone, we have 7.43 grams or only 91 per cent of the amount added. The results of the oxidation method (page 276) represent the expected 90 to 92 per cent of the amount added.

The reason for this loss on extraction is not altogether clear, but is probably due to an oxidation of some of the oxybutyric acid. We have frequently found in the ether extracts a substance which distills off without the addition of any oxidizing agent and which readily reacts in the cold with hypoiodate to form iodo-

¹⁷ *Loc. cit.*

form.¹⁸ The substance apparently is not derived from the ether or the plaster, and the preformed acetone and diacetic acid are of course driven off during the preliminary evaporation of the urine. Although the evidence is not conclusive, it is probable that the substance is acetone produced from an oxidation of a little of the oxybutyric acid during the dehydration with plaster or during the extraction.

It appears that Black's application of the extraction method and polarization of the extract usually gives practically correct results, but that the results are somewhat uncertain because they are influenced by the opposing errors of a levo-rotary substance, not oxybutyric acid, tending to give too high values, and on the other hand an occasional incomplete extraction and decomposition of some of the oxybutyric acid, tending to make the results too low.

The extraction method is very serviceable, although from our experience we prefer for most purposes the oxidation method, because the latter is quicker, requires less manipulation and apparatus, less urine, and especially for small amounts of oxybutyric acid is, with the correction, more accurate.

It is of interest that the parallel determination by the two methods, one of which determines both *d* and *l* forms of the acid, has given no evidence for the occurrence in diabetic urine of *d*-oxybutyric acid. The asymmetric formation of the levo oxybutyric acid, so far as indicated by available evidence, appears to be perfect.

SUMMARY.

1. The method for the determination of oxybutyric acid by oxidation to acetone with chromic acid is found to give uniformly about 90 per cent of theoretical values. The results obtained by the method must therefore be corrected by the addition of 10 per cent of the amount found.

2. A procedure for the isolation and purification of oxybutyric acid in the form of a new double salt of calcium and zinc is described.

3. Results by the oxidation method are compared with results obtained by Black's technique of the ether extraction method.

¹⁸ The iodoform-forming substance is usually lost during the removal of the ether on the water bath.

THE DETERMINATION OF ACETONE.

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(Received for publication, September 4, 1913.)

In the determination of β -oxybutyric acid through oxidation to acetone by the Shaffer bichromate method¹ with subsequent estimation of the acetone by Messinger's iodimetric titration,² difficulty was experienced in obtaining the theoretical amount of acetone from known amounts of pure β -oxybutyric acid. It was suspected that either the Messinger titration was inaccurate or that the acetone was incompletely recovered in the distillates.

Messinger in his original paper,³ found that in dilute aqueous solutions of acetone, slightly low results were obtained. Collischonn⁴ found that the Messinger method gave low results in very dilute acetone solutions. Geelmuyden⁵ using the Messinger method obtained satisfactory results on purified acetone in aqueous solutions, but found that, on distillation of such solutions, loss of acetone of from 5 to 10 per cent was unavoidable, even when ice-cooled receivers were used and the solutions were distilled almost to dryness. Denigès⁶ claims that the first quarter of the distillate from aqueous acetone solutions contains only about 90 per cent of the acetone present.

In view of the results cited above, it seemed desirable to investigate the accuracy of the Messinger titration and also to determine whether a dilute aqueous acetone solution could be distilled without loss. In addition I have tested the accuracy of the recently described acetone estimation of Scott-Wilson.⁷

¹ Shaffer: this *Journal*, v, p. 211, 1908.

² Messinger: *Ber. d. deutsch. chem. Gesellsch.*, xxi, p. 3336, 1888.

³ *Loc. cit.*

⁴ Collischonn: *Zeitschr. f. anal. Chem.*, xxix, p. 562.

⁵ Geelmuyden: *Ibid.*, xxxv, p. 503.

⁶ Denigès: *Ann. Pharm. de Bordeaux*, 1910.

⁷ Scott-Wilson: *Journ. of Physiol.*, xlii, p. 444.

The Messinger method.

A sample of acetone prepared from the bisulphite compound (Eimer and Amend) was further purified by distillation over potassium permanganate, and redistillation over fused calcium chloride. The product, which was anhydrous and free from aldehyde, was subjected to fractional distillation and two portions collected between 56° and 57°C.

Dilute aqueous solutions containing known amounts of this purified acetone were prepared as follows. Thin glass bulbs of 2 or 3 cc. capacity, and provided with a capillary side tube, were blown. These were weighed and then filled with acetone,⁸ sealed, and again weighed. Each bulb was introduced into a 2-liter glass stoppered volumetric flask nearly filled with water. The bulbs were broken under the surface of the water by a sharp blow from a glass rod; distilled water was then added to the mark and the contents thoroughly mixed.

Bulb I		Bulb II	
Bulb and acetone.....	3.1928	Bulb and acetone.....	2.5939
Bulb empty.....	0.7454	Bulb empty.....	0.9194
	<u>2.4474</u>		<u>1.6745</u>
Corr. for air displacement . .	0.0024	Corr. for air displacement . .	0.0017
Acetone.....	<u>2.4498</u>	Acetone.....	<u>1.6762</u>

In measuring out even such dilute solutions as the ones thus prepared, care was necessary in order to obviate loss of acetone. The solution was forced up into the pipette by air pressure from an atomizer bulb, the neck of the flask being closed by a double-holed rubber stopper. In delivering the solution the pipette was always under the surface of water in the receiving vessel. In this manner 25-cc. portions of the solutions prepared as above, were measured into 700-cc. Florence flasks containing each about 500 cc. of distilled water. A measured amount of standardized iodine solution was then run in, 10 cc. of 60 per cent sodium hydrate added, the flasks stoppered, shaken a little, and allowed to stand for five or ten minutes, after which 15 cc. of concentrated hydrochloric acid were added and the liberated iodine titrated with

⁸ The bulbs were warmed, and then the tips of side tubes dipped into acetone, so that on cooling the bulbs acetone rushed in.

standard sodium thiosulphate⁹ in the usual manner. The following results were obtained.

SOLUTION I.		cc.
Iodine solution added 50 cc.....		(49.8)
Thiosulphate.....		19.0
Iodine used up.....		30.8
Then since 1 cc. of $\frac{N}{10}$ iodine is used up by 0.968 mgm. of acetone		
30.8×0.968×102.8 per cent=30.64 mgm.		} Acetone.
Present by weighing..... 30.62 mgm.		

SOLUTION II.		cc.
Iodine solution added 50 cc.....	(49.8)	
Thiosulphate.....	28.6	
Iodine used up.....	21.2	
Then $21.2 \times 0.968 \times 102.8$ per cent = 21.09 mgm. }		
Present by weighing..... 20.95 mgm. }		Acetone.

The Messinger method, then, is accurate even in quite dilute solutions.

Distillation of acetone from dilute solutions.

It is frequently necessary to distil acetone solutions before making the final determinations. I have found, contrary to the results of Geelmuyden and Denigès,¹⁰ that if proper precautions are taken, acetone may be completely distilled off from even a dilute aqueous solution and entirely recovered in the distillate. Ten minutes' distillation is sufficient to accomplish this result, as is shown in the following experiment.

An acetone solution was used, 500 cc. of which when titrated by the Messinger method were found to contain 33.7 mgm. of acetone. This amount of solution was distilled from an 800-cc. Kjeldahl flask, using a block tin condenser connected with a glass delivery tube, the end of which dipped under the surface of about 50 cc. of water contained in the receiving flask. No ice cooling was used or found to be necessary. Distillations were continued for the length of time indicated, and acetone in the distillates immediately determined by the Messinger method.

⁹ The thiosulphate was standardized against pure potassium bi-iodate and also against bichromate and found to be 102.8 per cent of $\frac{N}{10}$. 50 cc. of the iodine solution were equivalent to 49.8 cc. of thiosulphate, on blank titrations.

¹⁰ *Loc. cit.*

Time distilled in minutes	Acetone in distillate mgm.
5	31.1
10	33.6
10	33.5
10	33.7
15	33.7
20	33.6
25	33.7
30	33.7

The anomalous results of Geelmuyden and of Denigès may possibly be explained by their failure to always have the end of the delivery tube dip under the surface of the liquid in the receiving flask.

The mercury cyanide method of Scott-Wilson.

Although the Messinger method gives correct results and is the most satisfactory for general use when considerable amounts of acetone are to be determined, yet it is not of sufficient delicacy to determine such small amounts of acetone as occur, for example, in a few cubic centimeters of blood.

A more delicate method is that described by Scott-Wilson.¹¹ This depends upon the precipitation of acetone as a keto-mercury-cyanide compound with subsequent determination of the mercury by titration with a standard sulphocyanate solution under prescribed conditions.

In carrying out the method, as described, several difficulties were encountered, and correct results were not obtained. With certain modifications of the procedure, however, I have found the method to be capable of considerable accuracy with exceedingly small quantities of acetone.¹²

The best results are obtained in the following manner:

Dilute solutions of pure acetone are run into an excess of the recently filtered reagent¹³ contained in small Erlenmeyer flasks, allowed to stand

¹¹ *Loc. cit.*

¹² The method is applicable only for quantities of acetone less than five milligrams.

¹³ The reagent is made up as follows: Mercuric cyanide, 10 grams; Sodium hydroxide, 180 grams; Water, 1200 cc. The solution is agitated in a flask and 400 cc. of a 0.7268 per cent solution of silver nitrate slowly run in. At least 30 cc. of the reagent must be taken for each milligram of acetone present.

twenty minutes and then filtered through an asbestos mat¹⁴ in a separable bottom Gooch crucible. By first filtering an aqueous suspension of talcum powder so as to partly close the pores of the filter, less difficulty is experienced in obtaining clear filtrates. In some cases the first portions of the filtrate are turbid and have to be refiltered. The precipitate is washed with cold water until the washings are free from silver.

With the aid of a pointed hooked glass rod the precipitate, mat, and crucible bottom are transferred to a 50-cc. beaker, any adhering particles of the precipitate being washed into the beaker with about 10 cc. of "acid mixture,"¹⁵ 1 cc. of $\frac{N}{5}$ potassium permanganate is added, the beaker covered with a watch glass, and the liquid boiled until colorless. More permanganate is then added a few drops at a time, until a persistent brown color is obtained which does not disappear on boiling for a couple of minutes. The brown color is then discharged by the addition of a few drops of strong yellow nitric acid. The greater the amount of acetone present the more permanganate is required, and it is essential to the accuracy of the method that an excess be added as indicated above, otherwise the results are low.

The beaker is cooled under the tap, 2 cc. of saturated ferric alum added, and a standard solution of potassium sulphocyanate (approximately 0.1 per cent) run in from a burette until a very faint pinkish brown color is obtained throughout the solution. The end point, which consists in the faintest trace of color, can be detected only when the titration is performed on a pure white surface. A control beaker with one drop excess of sulphocyanate should be at hand for comparison. A whole cubic centimeter of sulphocyanate may be run in after the end point is reached without very greatly darkening the shade.

In the calculation of results Scott-Wilson has assumed that the keto-mercury-cyanide compound has the formula $\text{HgCOC}_2(\text{HgCN})_4$ and that consequently 1 mgm. of mercury should be equivalent to 0.058 mgm. of acetone. He determined the value of the sulphocyanate solution in terms of mercury by titrating it against a known mercury solution. In applying this method to the estimation of pure acetone solutions he obtained results about 3 per cent too low. The error he attributed to loss of acetone by evaporation, or to impurities in the acetone. My results which follow, have led me to believe that the error is instead in the method of calculation.

The dilute acetone solution made up from Bulb I, and used as previously described for the Messinger titration was also used in this case. Twenty-five cubic centimeters of this solution were made up to 1 liter with distilled water, and 50 cc. of this latter

¹⁴ Filter paper cannot be used as the strong alkali quickly attacks it.

¹⁵ Nitric acid, 40 parts; Sulphuric acid, 5 parts; Water, 55 parts.

solution, containing 1.53 mgm. acetone, used for each determination. The acetone solutions were each run into 50 cc. of acetone reagent and the estimations carried out as described above.

On titration the following results were obtained, and calculations based on the value of the sulphocyanate solution made as indicated.

KSCN ¹⁶	ACETONE FOUND	ACETONE PRESENT	"ACETONE FACTOR"
cc.	mgm.	mgm.	
23.7 × 0.061.....	1.44	1.53	0.0646
24.1 × 0.061.....	1.47	1.53	0.0635
24.0 × 0.061.....	1.46	1.53	0.0637
23.6 × 0.061.....	1.44	1.53	0.0648

A second acetone solution was made up from the same stock solution from Bulb I, by diluting 25 cc. of this to 250 cc. with water. Of this latter solution 10 cc., containing 1.225 mgm. acetone, were used for each determination.

KSCN	ACETONE FOUND	ACETONE PRESENT	"ACETONE FACTOR"
cc.	mgm.	mgm.	
19.2 × 0.061.....	1.17	1.225	0.0638
18.6 × 0.061.....	1.13	1.225	0.0658

The results are uniformly low. The figures under the heading "acetone factor" represent the value by which each cubic centimeter of potassium sulphocyanate solution used should be multiplied in order to give correct results for the amount of acetone actually in the solution. The average of these values, which is 0.0644, is then to be taken as the true value of the sulphocyanate solution in terms of acetone. From the foregoing it is evident that the sulphocyanate solution cannot be standardized by its mercury equivalent, but that solutions of pure acetone of known strength, as determined by weighing or Messinger titration, can be used to advantage. The discrepancy in the results obtained by using the

¹⁶ The KSCN solution was standardized against a solution of mercuric nitrate, that had been analyzed for mercury by sulphide precipitation. 1 cc. of KSCN was found to be equivalent to 1.05 mgm. mercury, which from Scott-Wilson's formula, would correspond to 0.061 mgm. of acetone.

mercury equivalent as a basis of calculation may be explained by the possibly incorrect formula for the keto-mercury-cyanide compound or by the reaction not being a complete one.

Having thus standardized the sulphocyanate solution, a series of determinations on a different acetone solution was made. The solution used contained 0.172 mgm. of acetone per cubic centimeter, as determined by Messinger titration. Varying amounts of the solution and of acetone reagent were used in order to test the accuracy under different conditions.

ACETONE SOLUTION	ACETONE REAGENT	KSCN	ACETONE FOUND	ACETONE PRESENT
cc.	cc.	cc.	mgm.	mgm.
1	50	2.65×0.0644	0.170	0.172
1	50	2.85×0.0644	0.183	0.172
5	50	13.40×0.0644	0.863	0.860
5	50	13.50×0.0644	0.869	0.860
10	100	27.20×0.0644	1.75	1.72
10	100	26.80×0.0644	1.73	1.72
20	100	54.00×0.0644	3.48	3.44
20	100	54.40×0.0644	3.50	3.44

The method, then, gives accurate results with varying amounts of acetone and the accuracy is not affected by considerable amounts of acetone reagent in excess of the quantity required. As previously mentioned it is necessary to use at least 30 cc. of the acetone reagent for each milligram of acetone present, or expected to be present.

The acetone reagent is not affected by alcohol, but a precipitate forms with very small amounts of aldehydes, chlorides, hydrogen sulphide, or ammonia. In making determinations, therefore, the absence of these substances must be assured.

If the acetone solution is extremely dilute so that several hundred cubic centimeters are required to make a determination, the results have been found to be somewhat low. In such cases it is necessary to distil the acetone into a smaller volume of water, or better, directly into the acetone reagent. Boiling for ten minutes is sufficient to bring over all of the acetone, and the distillate need not amount to more than 100 cc.

The utilization of this method in the determination of acetone and of β -oxybutyric acid in blood and tissues appears in a subsequent paper.

SUMMARY.

1. The Messinger method for acetone estimation gives correct results.
2. The Scott-Wilson method gives accurate results only when certain modifications in the original procedure are made. It is applicable to very minute quantities of acetone.
3. In distilling a very dilute acetone solution, all of the acetone may be collected in the distillate within ten minutes.

NEPHELOMETRIC DETERMINATION OF MINUTE QUANTITIES OF ACETONE.

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(Received for publication, September 4, 1913.)

In order to determine very small amounts of acetone such as occur, for example, in a few cubic centimeters of normal blood, it is necessary to have a method more delicate than those at present in use.

As has been shown in the previous paper, the Scott-Wilson method is a delicate and accurate one for acetone determination, but it is not sufficiently delicate for the small amounts of acetone we wished to determine, so another method was devised.

The addition of acetone to a silver-mercury-cyanide solution gives rise to an abundant white nebulous precipitate. So delicate is the reaction for acetone that 0.01 mgm. is sufficient to cause a distinct opalescence in 50-100 cc. of solution. Further, the density of the opalescence, as measured by the nephelometer, has been found, within limits, to be proportional to the amount of acetone added. The details of the procedure are as follows:

The acetone solution, which must be free from ammonia, aldehyde or hydrogen sulphide, is distilled into an excess of the acetone reagent.¹ The delivery tube must dip under the surface of the

¹ The reagent is made up as follows: Mercuric cyanide, 10 grams; Sodium hydroxide, 180 grams; Water, 1200 cc. The solution is agitated in a flask and 400 cc. of 0.7268 per cent silver nitrate solution slowly run in. Immediately before use the reagent must be filtered through an asbestos mat, the pores of which have been partially occluded by previous filtration of a little talcum in water. At least 30 cc. of the reagent must be taken for each milligram of acetone present or expected to be present. A little experience enables one to tell by the density of the precipitate formed in the first couple of minutes' distillate the approximate amount of acetone present. A dense precipitate may call for the addition of more reagent to the receiving flask.

liquid in the receiving flask. The distillation is continued for about fifteen minutes or until the distillate measures from 75 cc. to 100 cc. After standing for about half an hour, the distillate is transferred to a graduated cylinder and diluted until an opalescence that can be conveniently read is obtained. The turbidity occasioned by 0.05 mgm. of acetone diluted to 100 cc. is a convenient strength for this purpose, although considerably smaller or larger amounts give good results. With heavy opalescence it is desirable after diluting to a certain volume, say 250 cc., to remove an aliquot portion with a pipette and dilute this appropriately. A solution containing a known amount of acetone² is distilled into an excess of reagent³ and this distillate which is to be used as the standard is diluted as above.

Comparisons of the turbidity of the unknown solutions with that of the standard are made in the nephelometer of Richards.⁴

The nephelometer as originally described may be improved by substituting the telescopic attachment of a Duboseq colorimeter for the eye piece instead of the plain brass tube used by Richards. A further modification consists in a partition between the two tubes. This was designed to eliminate reflections of light from one tube to the other.

Owing perhaps to inaccurate construction of the instrument the same solution when read in both tubes does not necessarily give identical readings. This source of error may be eliminated by making a series of readings, then reversing the tubes and making another series of readings, averaging the two ratios thus obtained; or more simply, as suggested by Kober,⁵ by reading the standard solution as an "unknown" and taking this value as the potential height of the standard solution.

As the suspensions slowly settle out, the readings should be made as quickly as possible after filling the tubes.

² A convenient stock solution contains about 0.03 mgm. acetone per cc. The strength of such a solution is determined by titration of 200 cc. by the Messinger method.

³ The solution cannot be added directly to the reagent as a lower result is obtained than when distilled.

⁴ Richards: *Zeitschr. f. anorgan. Chem.*, viii, p. 269, 1895; Richards and Wells: *Amer. Chem. Journ.*, xxxi, p. 235, 1904.

⁵ Kober: *this Journal*, xiii, p. 485, 1913.

The instrument is manipulated in a dark room, a small electric flash lamp being used to read the scale.

As originally pointed out by Richards⁶ the amounts of precipitate are not exactly inversely proportional to the scale readings. Kober⁷ has constructed a curve of correction for use with his modification of the nephelometer. When the two solutions for comparison are of nearly the same concentration, the correction is within the limits of observational error and may be disregarded. Further, by using Kober's equation for a correction curve it is seen that the difference between observed and corrected values becomes proportionately less with readings taken with greater depths of solution. If the unknown suspension is so diluted as to be not more than 20 per cent different from the standard and if comparisons are made with scale readings in the neighborhood of 50 mm. or 60 mm., no corrections are necessary.

In doing a series of determinations a single standard suspension is used and the various unknown suspensions are diluted in graduated cylinders to approximately the same opalescence. Little difficulty is experienced in thus obtaining suspensions differing from the standard by not more than 10 per cent.

It is to be mentioned that the nephelometer used was mechanically crude with no vernier and no ratchet and pinion attachments for adjusting the sliding jackets surrounding the tubes. Greater accuracy could possibly be obtained by using a modification of the Duboseq colorimeter. However, quite satisfactory results are possible as is shown below:

Solutions containing varying amounts of acetone were prepared by another member of the staff and determinations made by the writer on these solutions with the following results:

Acetone added	Acetone found
0.015	0.015
0.022	0.021
0.092	0.083
0.28	0.27
0.63	0.65
1.00	0.92
1.54	1.54

⁶ *Loc. cit.*

⁷ *Loc. cit.*

THE DETERMINATION OF β -OXYBUTYRIC ACID IN BLOOD AND TISSUES.

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(Received for publication, September 4, 1913.)

The Shaffer¹ method for the determination of β -oxybutyric acid is applicable to blood and tissue analysis.

Before applying the method, however, it is necessary to remove proteins and other disturbing substances. Proteins are removed by a modification of the Seegen² procedure of sodium acetate precipitation. The paired glucuronic acids, glucose and protein remnants are eliminated by a subsequent precipitation with basic lead acetate and ammonia.

The details of the method when large amounts of defibrinated blood or of tissues are available, are as follows:

A round-bottomed flask of 2 or 3 liters' capacity provided with a dropping funnel is connected with a condenser, the delivery tube of which dips beneath the surface of a little water contained in a 500-cc. receiving flask. The large flask contains 500 cc. of water, 3.5 cc. of glacial acetic acid and a little powdered talc. The liquid is raised to the boiling point. 100 cc. of blood diluted with 400 cc. of distilled water is then run in through the dropping funnel at such a rate that boiling does not cease.³ Distillation is continued until about 300 cc. have distilled. A very small amount of the liquid may occasionally foam over, but this is of no consequence on account of the subsequent redistillations. This distillate contains preformed acetone plus acetone from diacetic acid. Some

¹ Shaffer: this *Journal*, v, p. 211, 1908; Shaffer and Marriott, *ibid.*, xvi, p. 265, 1913.

² Seegen: *Centralbl. f. Physiol.*, vi, p. 604, 1893.

³ In using hashed organs the hash is all put into the dilute acetic acid before connecting up the apparatus. Care must be taken to shake the flask from time to time to prevent burning at the bottom.

ammonia may be present, hence redistillation with addition of a little dilute sulphuric acid is performed. A second redistillation⁴ after the addition of 20 cc. of 3 per cent hydrogen peroxide and a slight excess of alkali serves to destroy or hold back hydrogen sulphide, aldehydes, if any, and volatile acids. The final distillate is used for acetone determination by the Messinger titration in the usual manner.

The large flask is removed from the distilling apparatus, and while the contents are still hot, about 15 cc. of 20 per cent sodium carbonate solution are poured in, with stirring. When sufficient sodium carbonate has been added the dark grumous liquid changes to brown, and a flocculent precipitate settles leaving a clear straw colored supernatant liquid, of amphoteric reaction. The flask is held over a ring burner and the contents boiled for a minute or two, then allowed to cool and transferred to a graduated flask or cylinder and made up to 1000 cc. with water. The whole is thoroughly mixed and filtered through dry paper on a Büchner funnel. An aliquot portion (usually 700 cc.) is transferred to a graduated flask, 30 cc. basic lead acetate solution (U. S. P.) and 15 cc. strong ammonia added and the volume is made up to 1000 cc. The solution is mixed, allowed to stand awhile and filtered on a dry folded filter. 900 cc. of the water-clear filtrate are boiled to expel the greater part of ammonia and to concentrate to about 500 cc. This is cooled and sufficient dilute sulphuric acid added to precipitate the excess of lead present, the lead sulphate is filtered off, 30 cc. of 50 per cent sulphuric acid added and the whole transferred to a liter Kjeldahl flask provided with a dropping funnel. The contents of the flask are distilled and a solution of potassium bichromate or water is run in at such a rate that the liquid always retains some yellow color, and the volume remains between 400 and 500 cc. It is rarely necessary to add more than 0.5 gram of bichromate and an excess is to be avoided. Slow distillation is continued for two hours and 600 to 800 cc. of distillate collected, the precaution being taken that the tip of the delivery tube is always under the surface of water in the receiving flask. The distillate is redistilled with 20 cc. of peroxide and 5 cc. of 10 per cent sodium hydroxide, and the final distillate titrated by the Messinger method.

⁴ In a preceding paper it was shown (p. 283) that ten minutes' distillation is ample to distil off all acetone.

To test the accuracy of the method, determinations were made on fresh defibrinated beef blood to which had been added pure synthetic β -oxybutyric acid.⁵ The following results were obtained:

100 cc. blood alone: 8.4, 7.9, 8.8 mgm. oxybutyric acid; *average*, 8.3 mgm.

100 cc. blood to which had been added 82.08 mgm. of β -oxybutyric acid as determined on the pure solution: 90.9, 89.6 mgm. β -oxybutyric acid; *average found*, 90.2 mgm.; *amount present*, 90.3 mgm.

Another experiment on a different sample of blood gave the following results:

100 cc. blood alone: 7.2, 7.4 mgm. β -oxybutyric acid; *average*, 7.3 mgm.

100 cc. blood to which had been added 87.1 mgm. oxybutyric acid: 94.3, 92.3, 93.1, 93.9 mgm. oxybutyric acid; *average found*, 93.4 mgm.; *amount present*, 94.4 mgm.

50 grams muscle hash alone, 11.3 mgm. β -oxybutyric acid.

50 grams of same hash to which were added 173 mgm. β -oxybutyric acid: *found*, 182.1 mgm.; *present*, 184.3 mgm.

50 grams liver hash alone gave 16.5 mgm. β -oxybutyric acid.

50 grams of same hash to which were added 173 mgm. of β -oxybutyric acid: *found*, 186.5 mgm.; *present*, 189.5 mgm.

Method for small amounts of blood.

By determining the acetone in the distillates by the exceedingly delicate method of Scott-Wilson,⁶ I have been able to make satisfactory estimations of the acetone bodies in 10-cc. samples of blood, drawn directly from a vein.

The details of the method are as follows:

10 cc. of blood drawn from a superficial vein by a sterile graduated syringe are run into about 40 cc. of 0.5 per cent potassium oxalate solution.

An 800-cc. Kjeldahl flask, provided with a dropping funnel is connected with a condenser, the delivery tube of which dips beneath the surface of water in a receiving flask. The Kjeldahl flask contains 100 cc. of water and 1 cc. of glacial acetic acid. This is brought to a boil and the diluted blood slowly run in through the dropping funnel.

⁵ In the form of the purified calcium zinc double salt.

⁶ Scott-Wilson: *Journ. of Physiol.*, xlii, p. 444, 1911; Marriott: this *Journal*, preceding paper.

The liquid is kept boiling for about thirty minutes, after the last of the blood has been run in. The distillate is redistilled with a little dilute sulphuric acid and again with 20 cc. peroxide and a slight excess of alkali. The final distillate is caught in small Erlenmeyer flasks containing an excess of the Scott-Wilson "acetone reagent."⁷ The delivery tube must dip under the surface of the liquid, and it is not necessary to distil longer than ten minutes in order to get off all of the acetone. The resulting acetone-mercuric-cyanide compound is then filtered off on an asbestos mat in a Gooch crucible and acetone estimated as described in a previous paper.⁸ This represents acetone preformed and from diacetic acid.

The residue in the Kjeldahl flask is precipitated while still hot, with about 8 cc. of 10 per cent sodium carbonate, boiled a moment, filtered on a Büchner funnel and washed with hot water. To the clear filtrate are added 15 cc. of basic lead acetate (U. S. P.) and 10 cc. of strong ammonia. This precipitate is allowed to settle and then filtered off on a dry folded filter and the filtrate used for β -oxybutyric acid determination in the same way as described above for large quantities of blood, with the exception that the final distillate is caught in excess of "acetone reagent" and the estimation made by the modified Scott-Wilson method, previously described.

The following results were obtained with freshly drawn dog blood.

Results are expressed in terms of acetone obtained.

10 cc. of blood alone.

ACETONE PREFORMED AND FROM DIACETIC ACID		ACETONE FROM β -OXYBUTYRIC ACID	
	<i>mgm.</i>		<i>mgm.</i>
	0.03		0.32
	0.03		0.35
	0.03		0.34
Average 0.03		Average 0.33	

⁷ See preceding paper.

⁸ *Loc. cit.*

10 cc. of same blood to which had been added 1.74 mgm. acetone as oxybutyric acid.

ACETONE PREFORMED AND FROM DIACETIC ACID	ACETONE FROM OXYBUTYRIC ACID
mgm.	mgm.
0.06	2.12
0.06	2.14
0.06	2.16
Average..... 0.06	Average found... 2.14
	Amount present... 2.04

25-gram portions of hashed muscle of a fasting phlorhizinized dog gave the following results:

Messinger method...	$\left\{ \begin{array}{l} 7.4 \text{ mgm.} \\ 7.0 \text{ mgm.} \end{array} \right\}$	β -oxybutyric acid.
Scott-Wilson.....	$\left\{ \begin{array}{l} 7.5 \text{ mgm.} \\ 7.4 \text{ mgm.} \end{array} \right\}$	β -oxybutyric acid.

Nephelometric method.

By applying the nephelometer to the determination of the acetone occurring as such or as diacetic acid and also to that obtained from oxidation of the β -oxybutyric acid, it is possible to make a complete analysis using only from 2 to 5 cc. of blood.

For human work, blood is drawn from a superficial arm vein by means of a sterile syringe and run into about 50 cc. of 0.5 per cent potassium oxalate solution, contained in a small weighed flask.

The diluted blood is run into 100 cc. of boiling water acidified with 1 cc. of glacial acetic acid⁹ contained in an 800-cc. Kjeldahl distilling flask and the procedure carried out as described on pp. 295-6, with the exception that the amount of the precipitate in the mercury reagent is estimated by means of the nephelometer by the method given on pp. 289-90 in a preceding paper in this number.

The question may arise as to whether the substance giving a precipitate in the acetone reagent is really acetone. In this connection it is interesting to note that the results obtained on blood by my method agree closely with those obtained by the Messinger

⁹ Commercial varieties of acetic acid frequently contain substances which behave like acetone. Blank determinations should always be made and correction made accordingly.

iodimetric titration, in which iodoform was produced and identified microscopically. It is true that ammonia, chlorides, aldehydes, and hydrogen sulphide affect the reagent, but the absence of all of these is assured by the procedures adopted.

In view of the fact that the oxidation of oxybutyric acid by chromic acid gives only 90 per cent of the theoretical yield of acetone, as explained in a preceding paper, 10 per cent should be added to the results obtained by titration or by the nephelometer.

A few determinations follow, the results being expressed as milligrams of acetone per 100 grams of blood:

	Acetone plus dila- cetic acid	β -Oxybutyric acid
Normal dog.....	0.04	3.2
Normal dog.....	0.08	1.7
Normal dog.....	0.06	1.7
Normal child.....	0.06	4.4
Normal child.....	0.08	4.4
Dog, phlorhizinized.....	7.2	10.4
Child in coma.....	23.4	24.8
Child (following orthopedic operation)...	11.2	28.0

The methods as given above for the estimation of oxybutyric acid have the advantage over the usual optical methods, in that very much smaller quantities of β -oxybutyric acid may be determined with accuracy. The disturbing effect of optically active substances, such as sarcolactic acid, is eliminated. A further advantage in experimental work is that the methods are suitable for estimating optically inactive β -oxybutyric acid. When sufficiently large quantities of oxybutyric acid are present to permit a determination by the optical methods, a comparison of the results with those obtained by the method described in this paper shows the amounts of either dextro, levo, or inactive acid present.

This work was undertaken at the suggestion of Professor Shaffer, and I am greatly indebted to him for his active interest and valuable suggestions.

STUDIES OF THE ENDOGENOUS METABOLISM OF THE PIG AS MODIFIED BY VARIOUS FACTORS.

I. THE EFFECTS OF ACID AND BASIC SALTS, AND OF FREE MINERAL ACIDS ON THE ENDOGENOUS NITROGEN METABOLISM.¹

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That there is a fairly constant type of tissue metabolism resulting from the necessary cellular activity which has received the name endogenous metabolism, and which is in great measure independent of the nitrogen intake, is now generally accepted. That the group of metabolic end products of nitrogenous nature present in the urine of an animal whose diet contains no nitrogen shows relationships which are not found in urines under any other conditions is equally well established. It has become the practice, because of lack of knowledge to the contrary, to refer to endogenous metabolism as a single variety; that is, no effort has been made to resolve this type into factors. The most conspicuous and least variable known constituent of the group of endogenous end products of nitrogen metabolism is creatinine. Mendel and Rose² studying the conditions under which creatine is eliminated have shown conclusively that in fasting rabbits and dogs, or when the animals are on a diet of fat alone there is always an increase in the output of total creatinine (creatinine plus creatine). This rise is always attended by an increase in the total nitrogen output. They hold that creatinine is derived from creatine, and that those conditions which produce a carbohydrate hunger in the cells of the tissues, lead to excessive catabolism of the tissues and con-

¹ Published with the permission of the Director of the Wisconsin Experiment Station.

² This *Journal*, x, p. 247, 1911.

sequent liberation of creatine from the muscles. That further, the animal's power to convert creatine into creatinine, or to destroy it may thereby be exceeded and creatine may appear in the urine. Concomitant with this increased tissue catabolism goes of course an increased elimination of total nitrogen. In harmony with this theory are the data of Myers and Volovic³ published since the work reported in this paper was carried out. Working with rabbits they found that hyperthermia, whether caused by infection or by keeping the animal in an incubator, leads to an increased elimination of creatinine amounting to 36 per cent over the normal output. The view is expressed that creatinine elimination in fever still represents the normal endogenous metabolism which is here proceeding at an abnormal rate.

It would seem therefore that, if an animal were placed in a condition in which the endogenous type of protein catabolism alone prevailed, and then by some means this type of metabolism could be increased in amount we should anticipate a rise in the creatinine elimination, or an increased creatine output or both. A further question of interest which could be answered by such experiments is the relationship among the constituents of urines carrying nitrogen derived solely from "accelerated endogenous metabolism." Do these relationships always remain the same when exogenous catabolism is absent? We have attempted to throw some light on these questions by employing several methods of varying the intensity in endogenous metabolism in pigs living on a diet of carbohydrate, salts and water. The methods employed in the experiments described in this and in the two papers following, which it was assumed would stimulate protein metabolism, were (1) the addition of hydrochloric acid and acid salt mixtures to the starch diet; (2) the feeding of benzoic acid in the starch diet, and (3) the replacement of starch wholly or partially by fat.

In the present paper we will discuss the effects on endogenous metabolism of giving neutral, basic, and acid salt mixtures, and of free mineral acids with an adequate starch diet. Because of the ease of maintaining an animal during long periods under these experimental conditions we have deemed it best to employ the different methods of accelerating tissue catabolism on the same individuals so that all data would be better correlated and stand-

³ *This Journal*, xiv, p. 489, 1913.

ardized. For the sake of clearness the data presented in the following tables I to V will be discussed under separate titles.

In view of the exceptional advantages of the pig as an experimental animal it seemed possible to obtain new data concerning endogenous metabolism which can scarcely be gotten with any other type of animal, because it is very easy to place this animal in a condition where the endogenous type of catabolism alone prevails. One of us¹ has called attention to the fact that a pig will eat a diet of starch, water and salts during a long period (36 days) with no sign of disturbance of appetite or loss of weight. Attention was there called to the fact that after a time, which varied somewhat in different individuals, the total nitrogen output in the urine sank to a level where the creatinine N, which remains constant, forms about 18.5 per cent of the total. It was pointed out that this ratio could be used as an index to the maintenance requirements of the animal and would serve as a guide to enable one to feed comparable amounts of any substance to different animals. These observations have now been extended to a considerable number of additional animals and some further comment is necessary on this point. Further experience has shown that some pigs when kept for long periods on the starch, salt and water diet never reach so high a relationship of creatinine N to total N as 18.5 to 100. Others will drop the total N excretion regularly to a point where it is about five and a half times the nitrogen eliminated as creatinine. We have seen animals which even after sixty days on a nitrogen free diet had a ratio of creatinine N to total N of only 10 to 100. Whatever the ratio arrived at, it is very constant. Other pigs will have a ratio of anywhere between 10 and 18.5 to 100. In general it has been the most vigorous and healthy animals which have produced the urines having the highest percentage of creatinine N in proportion to total N. Pigs born in the fall in Wisconsin are frequently chilled so much as to lower their vitality and give them a tendency to pneumonia. It is especially in the fall pigs that we have failed to observe the total N to fall to five and a half times the N as creatinine. The reason for this is not clear. It seems certain to be associated in general with lack of vigor. In a number of instances pigs whose breathing gave evidence of lung affections have persisted in putting out in the urine more total

¹ McCollum: *Amer. Journ. of Physiol.*, xxix, p. 210, 1911.

N than corresponds to the creatinine. Pig No. 34 is an illustration. When given starch and an alkaline salt mixture the N as creatinine was only 9.4 per cent of the total. At autopsy, both lungs were found to be extensively affected with pneumonia.

It seems to be true, however, that the ratios between creatinine N and total N under these experimental conditions is not quite the same for all individuals. Thus in the case of No. 38 during the period on an alkaline salt mixture and starch the per cent of the total N as creatinine was 14.8. At the end of the experiment the animal was killed and examined but microscopically nothing pathological was apparent. If creatinine is a product of the metabolism of muscle tissue alone some variation in the relation between the total end products of tissue catabolism, and creatinine may be due to variation in the relative sizes of the organs as compared to the total amount of muscle tissue in the body. More experimental data showing such relationships in a considerable number of animals together with a careful study of the composition of the urines will be necessary to throw light on this question.

In an experience with more than thirty pigs it has been found that, the year through, probably three-fourths of all pigs of the sizes employed by us in experimental work, will on a liberal carbohydrate diet come to a stage where the creatinine N will make up about 18.5 per cent of the total, and this ratio can be employed to advantage for the calculation of the lowest level of protein metabolism of which the animal is capable. We have not seen the percentage rise above 18.5 per cent except in a single case formerly reported⁵ and this was possibly due to error in the analytical work. Another factor of importance in determining the ratio of creatinine N to total N after a considerable period on the starch diet is the character of the salt mixture supplied. We have repeatedly observed that the lowest level of total-N output is reached only when the salt mixture supplied has an excess of basic over acid radicals. This is due to the fact that when the diet is acid, ammonia is eliminated in quantities sufficient to neutralize the acids present in the diet and this ammonia N is, to a considerable extent, derived from additional protein destruction over what would take place if these acids were not present. This point will be discussed further later on.

⁵ McCollum: *Loc. cit.*

In all our experiments agar-agar was given in amounts sufficient to lead to regular evacuation of the intestine so the disturbing factor of the absorption of putrefaction products was kept as low as possible. There is always a regular loss of appreciable amounts of nitrogen derived from the secretions of the tract, and if these residues are not promptly eliminated, some of the nitrogen from this source will be absorbed and eliminated in the urine, changing in some degree the typical relationships between the creatinine N and total N.⁶

EFFECTS OF ACID AND BASIC SALTS AND OF FREE MINERAL ACIDS UPON THE ENDOGENOUS NITROGEN METABOLISM.

That neutrality be maintained in the blood and tissues is a fundamental condition of life. It is therefore essential that acid radicals, either ingested or of metabolic origin be neutralized. If acidosis obtains both the fixed cations and ammonia take part in the neutralization. With a normal nitrogen intake the ammonia of the urine under these conditions has been observed to rise while the urea-nitrogen is correspondingly decreased. What would be the result if the metabolism were of the endogenous type alone?

Experimental.

The animals employed in these experiments were young pigs brought to their lowest level of nitrogen elimination through several weeks of starch feeding. They were confined in special cages and the urine collected daily according to the methods of McCollum and Steenbock.⁷ Upon these samples daily analyses for total N, creatinine N, creatine N, ammonia N and urea N were made. Sulphur determinations were also made during two periods. In the case of the urea N the Benedict-Gephardt method was used rather than the Folin method as a matter of convenience. The small error involved in the use of this method is of no consequence for the purpose of this investigation.

In Table I are found the data for pig 34. The starch diet was begun on January 10 and analyses were commenced with the urine

⁶ Compare the papers of Folin and Denis: this *Journal*, xi, p. 167, 1912; Osborne and Mendel: Bulletin of the Carnegie Institution of Washington, No. 156, Part I, p. 39, 1911; Mendel and Fine: this *Journal*, xi, p. 13, 1912.

⁷ Research Bulletin No. 21, Wisconsin Experiment Station, 1912.

TABLE I.
 Pig No. 34: Weight, 31.5 pounds. Starch feeding started Jan. 10.
Distribution of nitrogen in urine.

PERIOD	DATE	VOL. OF URINE cc.	TOTAL N gms.	UREA N gms.	AMMONIA N gms.	CREATININE N gms.	CREATINE N gms.	REST N gms.	PER CENT OF TOTAL NITROGEN				RATION
									Urea	Ammonia	Creatinine	Creathine	Rest
I	Feb. 24	1820	1.17	0.69	0.230	0.118		0.132	59.0	19.7	10.1		11.3
	25	1980	1.27	0.80	0.250	0.091		0.120	63.0	19.7	7.2		10.2
	26	1570	1.21	0.72	0.265	0.091		0.134	59.5	21.9	7.5		11.1
	27	1360	1.27	0.81	0.178	0.080		0.202	63.8	14.0	6.4		15.9
II	28	1320	1.55	1.10	0.104	0.093		0.263	70.6	6.7	6.0		16.8
	Mar. 1	1510	1.11	0.80	0.087	0.085		0.138	72.1	7.8	7.7		12.4
	2	1550	1.04	0.69	0.090	0.090		0.170	66.4	8.7	8.7		16.4
	3	1530	0.95	0.69	0.080	0.099		0.081	72.6	8.4	10.4		8.5
	4	1460	1.09	0.74	0.108	0.084		0.158	67.9	9.9	7.7		14.5
	5	1800	1.15	0.81	0.095	0.123		0.122	70.5	8.3	10.7		10.6
	6	1600	0.83	0.51	0.076	0.094		0.150	61.5	9.2	11.3		18.1
	7	1630	0.92	0.64	0.060	0.091		0.129	69.6	6.5	9.9		14.0
	8	1680	0.90	0.52	0.115	0.085		0.180	57.8	12.8	9.4		20.0
	9	1700	0.98	0.53	0.098	0.091		0.261	54.1	10.0	9.3		26.6
	10	1760	0.90	0.50	0.093	0.080		0.227	55.6	10.3	8.9		25.2
	11	1240	0.73	0.39	0.082	0.064		0.194	53.5	11.2	8.8		26.6
III	12	1210	1.31	0.85	0.152	0.081		0.227	64.9	11.6	6.2		17.3
	13	1530	1.48	0.86	0.322	0.082		0.216	58.2	21.8	5.5		14.6
	14	1400	1.23	0.70	0.330	0.078		0.122	56.9	26.7	6.3		9.9
	15	1600	1.11	0.59	0.220	0.085		0.215	53.1	19.8	7.7		19.4

Starch, 60 cal. per kgm.
 Neutral salts (II), 10
 gms.

TABLE I.—Continued.

IV	16	850	1.52	0.64	0.469	0.089	0.322	42.2	30.9	5.9	21.2	Starch, lard, 60 cal. per kgm. Neutral salts (II), 10 gms.		
	17	860	1.38	0.69	0.386	0.104	0.200	50.0	28.1	7.5	14.5			
	18	560	1.16	0.57	0.305	0.083	0.202	49.1	26.3	7.2	17.4			
	19	1090	1.18	0.55	0.358	0.096	0.029	0.147	46.6	8.1	2.5		12.5	
	20	820	1.28	0.60	0.361	0.087	0.027	0.205	46.8	6.8	2.1		16.0	
	21	910	1.62	0.66	0.320	0.089	0.030	0.521	40.7	5.5	1.9		32.2	
	22	660	1.25	0.64	0.348	0.084	0.178	51.2	27.8	6.7	14.3			
	23	390	1.36	0.84	0.264	0.079	0.055	0.122	61.8	5.8	4.0		9.0	
	24	830	1.00	0.58	0.206	0.101	0	0.113	58.0	10.1	0		11.3	
V	25	790	1.78	1.10	0.354	0.089	0.077	0.160	61.8	20.2	4.3	9.0	Butter fat, 50 cal. per kgm. Neutral salts (II), 10 gms.	
	26	400	1.03	0.60	0.275	0.070	0	0.085	58.2	26.7	6.8	0		8.2
	27	360	1.13	0.65	0.268	0.057	0.015	0.142	57.5	23.7	5.0	1.2		12.6
	28	560	1.55	0.90	0.380	0.082	0.035	0.153	58.0	24.5	5.3	2.3		9.9
	29	400	1.50	0.84	0.390	0.060	0.081	0.129	56.0	26.0	4.0	5.4		8.6
	30	480	1.34	0.74	0.364	0.066	0.043	0.127	55.2	27.2	4.9	3.2		9.5
	31	660	1.45	0.80	0.396	0.070	0.058	0.126	55.2	27.3	4.8	4.0		8.7
Apr.	1	370	1.13	0.62	0.290	0.055	0.031	0.134	54.8	25.7	4.9	2.7		11.9
	2	520	1.28	0.67	0.366	0.058	0.051	0.135	52.4	28.6	4.5	4.0		10.5
	3	340	1.06	0.56	0.280	0.060	0.024	0.136	52.8	26.4	5.7	2.3	12.8	
	4	500	0.83	0.41	0.254	0.046	0.013	0.107	49.4	30.6	5.5	1.6	12.9	
Summary. Averages by periods.														
I-4 days.....	1.23	0.76	0.231	0.095	0.149	61.8	18.7	7.7	12.1	Starch, NaCl.				
II-11 days.....	0.96	0.62	0.089	0.090	0.165	64.6	9.3	9.4	17.2	Starch, alk. salts.				
III-4 days.....	1.28	0.75	0.255	0.082	0.195	58.6	19.9	6.4	15.2	Starch, neutral salts.				
IV-9 days.....	1.31	0.64	0.334	0.090	0.028	0.223	48.8	25.5	6.9	2.1	17.0	Starch, lard, neutral salts.		
V-11 days.....	1.28	0.72	0.329	0.065	0.039	0.130	56.2	25.7	5.1	3.0	10.2	Fat, salts.		

TABLE II.
 Pig No. 38: Weight, 39 pounds. Starch feeding started Feb. 21.
Distribution of nitrogen in urine.

PERIOD	DATE	VOL. OF URINE cc.	TOTAL N gms.	UREA N gms.	AMMONIA N gms.	CREATININE N gms.	CREATININE + UREA N gms.	HEAT N gms.	PER CENT OF TOTAL N				RATION
									Urea	Ammonia	Creatinine	Rest	
I	Mar.	17	1500	1.94	0.86	0.576	0.166	0.338	44.4	29.7	8.6	17.4	Starch, 90 cal. per kgm. salts (II), 10 gms. Neutral
		18	1400	2.03	0.75	0.588	0.135	0.557	37.0	29.0	6.7	27.4	
		19	1580	1.53	0.70	0.467	0.131	0.232	45.7	30.5	8.6	15.2	
		20	1800	1.77	0.79	0.562	0.188	0.230	44.6	31.8	10.6	13.0	
		21	1570	1.43	0.67	0.376	0.143	0.241	46.8	26.3	10.0	16.8	
		22	1580	1.58	0.62	0.530	0.188	0.242	39.2	33.5	11.9	15.3	
		23	1640	1.35	0.53	0.406	0.171	0.243	39.3	30.2	12.7	18.0	
		24	1840	1.50	0.57	0.486	0.175	0.269	37.9	32.4	11.7	17.9	
		25	1700	1.45	0.44	0.626	0.159	0.225	30.4	43.1	11.0	15.5	
		26	1520	1.49	0.62	0.425	0.186	0.259	41.6	28.5	12.5	17.4	
		27	1320	1.31	0.55	0.369	0.132	0.259	41.9	28.2	10.1	19.8	
		28	1740	1.53	0.60	0.543	0.186	0.201	39.2	35.5	12.2	13.1	
		29	2140	1.20	0.59	0.394	0.155	0.061	49.1	32.8	12.9	5.1	
II	Apr.	30	1940	1.01	0.54	0.109	0.191	0.170	53.4	10.8	18.9	16.8	Starch, 90 cal. per kgm. salts (IV), 10 gms. Alkaline
		31	1470	0.86	0.52	0.071	0.160	0.109	60.4	8.3	18.6	12.7	
		1	1740	1.20	0.75	0.070	0.171	0.208	62.5	5.8	14.2	17.4	
		2	1880	1.05	0.66	0.060	0.134	0.190	62.8	5.7	12.8	18.7	
		3	1730	1.24	0.68	0.152	0.185	0.223	54.8	12.3	14.9	18.0	
		4	1950	1.01	0.51	0.156	0.166	0.178	50.4	15.4	16.4	17.6	
		5	1780	1.11		0.085	0.171			7.7	15.4		
		6	1780	1.14	0.68	0.085	0.162	0.213	59.6	7.5	14.2	18.7	
		7	1830	1.02	0.60	0.088	0.155	0.177	58.8	8.6	15.2	17.4	

	10	1760	1.10	0.75	0.042	0.141	0.157	68.2	3.8	12.8	14.3	
	11	1740	1.06	0.72	0.070	0.131	0.139	67.9	6.6	12.4	13.1	
	12	1900	1.08			0.158				14.6		
III	13	920	1.26	0.82	0.088	0.133	0.219	65.0	7.0	10.6	17.4	Starch-fat, 75 cal. per kgm. Alkaline salts (III), 10 gms.
	14	570	1.92	1.32	0.123	0.163	0.314	68.8	6.4	8.5	16.4	
	15	920	1.71	1.09	0.221	0.143	0.256	63.8	12.9	8.4	15.0	
	16	990	1.54	0.95	0.127	0.119	0.344	61.7	8.2	7.7	22.3	
	17	860	1.71	1.14	0.248	0.172	0.150	66.7	14.5	10.1	8.8	
	18	820	1.63	1.03	0.236	0.156	0.208	63.2	14.5	9.6	12.8	
	19	} urine con-		taminated								
	20											
	21	700	1.39	0.85	0.201	0.129	0.210	61.1	14.4	9.3	15.1	
	22	770	1.57	1.01	0.117	0.192	0.251	64.3	7.5	12.2	16.0	
	23	950	1.05	0.67	0.076	0.121	0.117	183	63.8	7.2	11.5	17.4
	24	1010	0.94	0.54	0.097	0.120	0.130	183	57.4	10.3	12.8	19.5
	25	970	1.32	0.89	0.127	0.146	0.157	67.4	9.6	11.1	11.9	
	26	840	1.32	0.79	0.181	0.133	0.136	216	59.8	13.7	10.1	16.4
	27	710	1.28	0.83	0.102	0.115	0.124	233	64.8	8.0	9.0	18.2
	28	410	1.00	0.62	0.115	0.085	0.095	180	62.0	11.5	8.5	18.0
	29	800	1.91	1.23	0.262	0.174	0.324	61.8	13.2	8.8	16.3	
IV	30	360	1.22	0.73	0.127	0.114	0.105	249	59.9	10.4	9.3	20.5
	May 1	330	1.33	0.89	0.129	0.107	0.118	204	66.9	9.7	8.0	15.4
	2	540	1.52	1.04	0.087	0.120	0.137	273	68.4	5.7	7.9	18.0
	3	310	0.94	0.60	0.086	0.100	0.097	154	63.8	9.1	10.5	16.4
	4	370	1.55	1.03	0.190	0.111	0.100	219	66.4	12.3	7.2	14.2
	5	290	1.41	0.83	0.220	0.100	0.101	260	58.8	15.6	7.1	18.4
Summary. Averages by periods.												
I-13 days.....			1.55	0.64	0.488	0.163	0.258	41.3	31.5	10.5	16.7	Starch, neutral salts.
II-14 days.....			1.09	0.65	0.089	0.161	0.183	59.6	8.2	14.8	16.8	Starch, alkaline salts.
III-17 days.....			1.44	0.92	0.155	0.140	0.229	63.9	10.8	9.7	15.9	Starch-fat, alkaline salts.
IV- 6 days.....			1.33	0.85	0.140	0.109	0.227	63.8	10.5	8.2	17.0	Fat, alkaline salts.

of February 24. In the first period of four days an ample starch ration with sodium chloride was given. Since no alkaline salts were fed it was necessary for the animal to neutralize the metabolic sulphuric and phosphoric acids by alkali produced within the body. The ammonia elimination is consequently high, being about 19 per cent of the total N excreted, which averaged 1.23 grams per day. (A discussion of the creatinine metabolism will be reserved until later.) There follows a period of eleven days in which a similar starch ration is fed, but with a salt mixture which was of alkaline character (salt mixture I, p. 315). On the first day there was a slight increase in nitrogen elimination, followed immediately by a decrease which continued at the lower level throughout the period. The ammonia N sank to about one-third that of the previous period. It is to be especially noted that there was a distinct drop in the total N. A period of four days followed, in which the alkaline salt mixture was exchanged for an approximately neutral one (salt mixture II). The total N and ammonia N again increased. While the changes are small in themselves they form a large per cent of the total. It is believed that deductions may legitimately be made from such variations, since the experimental error which is of the magnitude 0.01 to 0.02 gram nitrogen, is small compared with the amounts of nitrogen eliminated daily. No exogenous nitrogen was present, and fluctuations have a significance as indicating actual quantitative changes in the metabolic processes.

The experimental work was begun upon pig No. 38 after the animal had received the starch diet for twenty-four days. During the first period of thirteen days a nearly neutral salt mixture was fed (salt mixture II). Under these conditions the ammonia production was high, averaging 0.488 gram, while the total N gave a daily average of 1.55 grams. During the succeeding period of two weeks, the salt content of the ration was changed to one of markedly basic character (salt mixture III). As is shown in Table II the average daily elimination of total N dropped from 1.55 grams to 1.09 grams, the ammonia from 0.488 gram to 0.089 gram, while the urea N and creatinine N remained constant. The very close agreement of the results for urea N in these two periods may be due to coincidence, but there may fairly be deduced from the averages the conclusion that an additional amount of protein has been catabolized in response to the acid character of the diet. One is

impressed with the fact that the organism was apparently not able to utilize the nitrogen of the urea fraction to neutralize the acidity and thus prevent an increased nitrogen elimination. There was no change in the creatinine output and so in accordance with the present conception of protein metabolism the additional protein destruction must have been derived from other sources than muscle tissue.

To demonstrate this point more conclusively one animal, No. 39, was fed 10 cc. of hydrochloric acid (1:4) each day during a period of five days (Table IV). The total N increased from an average of 2.86 grams to 4.03 grams, while the creatinine N gave an average in one period of 0.437 gram and in the other 0.424 gram. The probability that this "extra nitrogen" was derived from some tissues other than muscle is further supported by the observations upon the neutral sulphur in the urine of pig 38 during the different periods (Table III). The neutral sulphur remained con-

TABLE III.

Fig 38.

Neutral sulphur in urine.

PERIOD	DATE	VOL. OF URINE	TOTAL N	NEUTRAL S	RATION
		cc.	gms.	gms.	
I	Mar. 19	1580	1.53	0.024	Starch.
	20	1800	1.77	0.022	Neutral salts.
	21	1570	1.43	0.028	
	23	1640	1.35	0.024	
	24	1840	1.50	0.024	
	25	1700	1.45	0.026	
	26	1520	1.49	0.029	
	27	1320	1.31	0.034	
II	Apr. 1	1740	1.20	0.016	Starch, alkaline salts.
	2	1880	1.05	0.019	
	3	1730	1.24	0.021	
	4	1950	1.01	0.023	
	5	1780	1.11	0.030	
	6	1780	1.14	0.033	
	7	1830	1.02	0.035	

Summary. Averages by periods.

I. 8 days.....	1.48	0.026
II. 7 days.....	1.11	0.025

TABLE IV—Continued

III	Apr. 26	3240	5.44	3.03	0.519	0.648	1.243	55.7	9.5	11.9	22.9	Same ration + 10 gms. benzoic acid daily.
	27	3230	2.71	0.85	0.336	0.478	1.046	31.4	12.4	17.7	38.6	
	28	3340	2.46	0.62	0.241	0.460	1.139	25.4	9.8	18.7	46.4	
	29	2580	1.69	0.54	0.124	0.440	0.586	31.9	7.3	26.0	34.6	
	30	3060	2.20	0.48	0.245	0.524	0.951	21.8	11.1	23.8	43.2	
	May 1	2840	2.02	0.47	0.205	0.426	0.919	23.3	10.2	21.1	45.6	
	2	2980	2.31	0.52	0.167	0.576	1.047	22.5	7.2	25.0	45.4	
IV	3	2370	2.56	0.49	0.265	0.490	1.315	19.2	10.4	19.2	51.4	Same ration + 16 gms. benzoic acid daily.
	4	2990	2.87	0.55	0.430	0.478	1.412	19.2	15.0	16.7	49.2	
	5	2960	2.89	0.52	0.402	0.493	1.475	18.0	14.0	17.1	51.0	
	6	3220	3.30	0.69	0.438	0.380	1.792	20.9	13.3	11.5	54.4	
	7	2820	2.69	0.50	0.384	0.342	1.464	18.6	14.3	12.7	54.4	
	8	3240	3.96	0.57	1.140	0.438	1.812	14.4	28.8	11.1	45.8	
	9	2660	3.96	0.49	1.280	0.404	1.786	12.4	32.4	10.2	45.2	
V	10	2450	3.88	0.62	1.220	0.426	1.614	16.0	31.4	11.0	41.6	Starch, salts (II), 10 gms. HCl (1:4), 10 cc. benzoic acid, 16 gms. daily.
	11	2400	4.28	0.43	1.780	0.450	1.620	10.1	41.6	10.5	37.8	
	12	1840	4.09	0.58	1.780	0.404	1.326	14.2	43.5	9.9	32.4	
<i>Summary. Averages by periods.</i>												
I-12 days.....			2.56	1.43	0.206	0.488	0.424	55.9	8.0	19.1	16.6	Starch, alkaline salts.
II-4 days.....			2.63	1.29	0.206	0.456	0.681	49.1	7.8	17.4	25.9	Starch, etc., benzoic acid, 4 gms.
III-7 days.....			2.23	0.58	0.220	0.484	0.948	26.0	9.9	21.7	42.5	Starch, etc., benzoic acid, 10 gms.
IV-5 days.....			2.86	0.55	0.384	0.437	1.492	19.2	13.4	15.3	52.1	Starch, etc., benzoic acid, 16 gms.
V-5 days.....			4.03	0.54	1.44	0.424	1.632	13.4	35.8	10.5	49.4	Starch, benzoic acid, HCl, 16 gms.

* This includes hippuric acid N.

stant whether the diet contained a neutral or an alkaline salt mixture.

It might be suggested that the extra nitrogen catabolized with the acid ration had its origin in the liver and that no extra muscle protein was decomposed, which would be in harmony with the constancy of creatinine elimination in the different periods. Hedin⁸ has shown that outside the body an acid reaction causes increased proteolysis in liver tissue. Arinkin⁹ has obtained similar results. If analogous conditions obtain in the functioning organ the increased nitrogen with constant creatinine elimination would be explained. In this connection it may be recalled that in certain pathological conditions, as in phosphorus poisoning, with its accompanying degeneration of the liver, an increased nitrogen elimination takes place without increased creatinine.¹⁰ Schryver,¹¹ as a result of his studies upon the autolysis of organs, has advanced the hypothesis that the stability of the liver is the result of the mass action of three sets of bodies, the tissues, the metabolites or bodies derived therefrom, and the autolytic enzymes. An acid reaction causes increased activity of the autolytic enzymes, with the degradation of protein. The resultant amino-acids are the sources of the ammonia necessary to restore neutrality. In cases of starvation or of low nitrogen intake these proteolytic enzymes serve automatically to adjust the destruction of tissues to the requirements of acid neutralization. If this conception be correct we should reason that a nitrogen-free ration of sufficiently alkaline reaction would reduce the endogenous output to a level lower than could be obtained by any other combination of food ingredients. Conversely we should assume that the feeding of a non-oxidizable acid would accelerate the proteolysis. Pathologically, any conditions which would increase the acid formation within the body should also increase the liver autolysis and consequently the amount of nitrogen eliminated. Schryver has cited the examples of insufficient oxygen intake and of phosphorus poisoning. In each instance the diminished oxidation results in the accumulation of intermediate prod-

⁸ *Festschrift für Hammarsten*, 1906.

⁹ *Zeitschr. f. physiol. Chem.*, liii, p. 192, 1907.

¹⁰ Mendel and Rose: this *Journal*, x, pp. 213-264, 1911, give a full discussion of the literature on this subject.

¹¹ *Biochem. Journ.*, i, p. 123, 1906.

Fig 43: Weight about 49 pounds.
Distribution of nitrogen in the urine.

PERIOD	DATE	VOLUME OF URINE	TOTAL N	UREA N	AMMONIA N	CREATININE N	UNDETERMINED N	PER CENT OF TOTAL N				RATION
								Urea	Ammonia	Creatinine	Rest N	
I	May	18	2.30	0.91	0.94	0.226	0.224	39.6	40.8	9.8	9.7	Neutral salts (II), 10 gms. + 10 cc. HCl (1:4); 75 cal. per kgm. as starch.
		19	1.74	0.72	0.69	0.175	0.155	41.4	39.6	10.1	8.9	
		20	1.89	0.90	0.70	0.234	0.196	44.3	34.5	11.5	9.7	
		21	2.08	0.80	0.73	0.213	0.287	39.4	36.0	10.5	14.1	
		22	2.02	0.77	0.72	0.196	0.234	40.1	37.5	10.2	12.2	
		23	1.93	0.83	0.93	0.236	0.274	36.6	41.0	10.4	12.1	
		24	1.71	0.71	0.62	0.192	0.188	41.5	36.2	11.2	11.0	
		25	2.04	1.81	0.75	0.229	0.191	41.4	35.3	12.7	10.6	Alkaline salts (IV), 10 gms.; 75 cal. per kgm. as starch.
		26	2.20	1.86	1.01	0.211	0.239	54.3	21.5	11.3	12.8	
		27	1.44	0.83	0.25	0.180	0.180	57.6	17.3	12.5	12.5	
II	June	28	1.59	1.68	1.02	0.245	0.165	60.7	14.9	14.6	9.8	
		29	1.41	0.88	0.19	0.169	0.171	62.4	13.5	12.0	12.1	
		30	2.08	1.51	0.93	0.198	0.102	61.6	18.5	13.1	6.8	
		31	2.19	1.44	0.95	0.202	0.078	66.0	14.6	14.0	5.4	
		1	1.78	1.13	0.25	0.214	0.186	63.4	14.0	12.0	10.4	
		2	1.47	0.99	0.19	0.194	0.086	67.3	12.9	13.2	6.5	
		3	1.60	1.77	0.85	0.199	0.551	48.0	9.6	11.2	31.1	75 cal. per kgm. as starch; alkaline salts (IV), 10 gms. + 5 gms. benzoic acid + 2 gms. Na ₂ CO ₃ .
		4	2.09	1.92	0.84	0.230	0.650	43.7	10.4	12.0	33.8	
		5	2.18	1.52	0.57	0.142	0.648	37.5	10.5	9.3	42.6	
		6	2.42	1.99	0.70	0.187	0.913	35.2	9.5	9.4	45.8	Alkaline salts (IV), 10 gms. + 10 gms. benzoic acid + 2 gms. Na ₂ CO ₃ ; 75 cal. per kgm. as starch.
III	June	7	1.86	1.68	0.42	0.189	0.941	25.0	7.7	11.3	56.0	
		8	2.08	1.66	0.35	0.192	0.968	21.1	9.0	11.6	58.3	
		9	2.27	1.65	0.31	0.188	0.972	18.8	10.9	11.4	58.8	
		10	2.31	1.59	0.29	0.154	0.996	18.2	9.4	9.7	62.6	
IV	July	11	2.00	0.81	0.76	0.21	0.22	40.5	38.0	10.5	11.0	Neutral salts + HCl.
		12	1.60	0.94	0.29	0.21	0.16	58.8	18.1	13.1	10.0	Alkaline salts.
		13	1.74	0.75	0.18	0.19	0.62	43.0	10.3	10.9	35.6	Same + 5 gms. benzoic acid.
		14	1.65	0.34	0.15	0.18	0.97	20.6	9.1	10.9	58.8	Same + 10 gms. benzoic acid.
		15	1.65	0.34	0.15	0.18	0.97	20.6	9.1	10.9	58.8	
		16	1.65	0.34	0.15	0.18	0.97	20.6	9.1	10.9	58.8	
		17	1.65	0.34	0.15	0.18	0.97	20.6	9.1	10.9	58.8	
		18	1.65	0.34	0.15	0.18	0.97	20.6	9.1	10.9	58.8	
		19	1.65	0.34	0.15	0.18	0.97	20.6	9.1	10.9	58.8	
		20	1.65	0.34	0.15	0.18	0.97	20.6	9.1	10.9	58.8	

Summary. Averages by periods.

ucts of combustion (*e.g.*, lactic acid), and it has been demonstrated experimentally that there is also increased nitrogen catabolism. In pathological cases where the liver becomes strongly acid its degeneration is rapid.

The experimental data reported in this paper are in harmony with this hypothesis. The constancy of the creatinine N as contrasted with the marked changes in the total N finds its explanation in the sources of the nitrogen fractions. According to the present theories of protein metabolism, practically all of the creatinine N originates from the muscle tissue, but an acid acceleration of catabolism does not materially affect the creatinine output. It seems probable that the extra nitrogen eliminated under the influence of acid is derived from the liver. If this is the case we should not expect an increase in creatinine output to follow even a marked increase in the total N elimination in acidosis. The experimental data available indicate that the endogenous metabolism of certain tissues can be selectively accelerated by the introduction of acid salts and of hydrochloric acid into the diet.

Two possible explanations seem available for the great excess of nitrogen eliminated by pigs under the influence of acids. It may be assumed that the animal cannot use the nitrogen, which would appear as urea if the diet contained alkaline salts in excess, for the production of ammonia necessary to maintain neutrality in the body. It is also possible that the nitrogen of the urea fraction is utilized in the first instance to produce ammonia and that the nitrogen catabolism of the tissues is stimulated by the presence of the ammonium salts thus formed. Our data do not afford an answer as to the correctness of either of these views. Since these experiments were carried out Underhill¹² has published results which seem to indicate that ammonium salts do stimulate endogenous metabolism. There was a large exogenous factor in his experiments and the available data do not seem to warrant a conclusion as to the correctness of the view when the endogenous type of metabolism alone prevails.

¹² Underhill: *this Journal*, xv, p. 327, 1913.

SUMMARY OF CONCLUSIONS.

1. Data are presented which show that the endogenous metabolism of the pig reaches its lowest level when the animal has an abundant supply of carbohydrates together with a salt mixture of an alkaline character.

2. The total output of nitrogen derived from endogenous sources can be greatly increased without changing the output of creatinine.

3. The additional nitrogen which is eliminated on an acid over what appears on an alkaline diet is in the form of ammonia. The animal is not able to use the nitrogen of the urea fraction to neutralize the acids present in the diet, but draws additional nitrogen from the tissues for ammonia production.

Composition of salt mixtures.

Salt mixture I.

	<i>per cent</i>
NaCl.....	0.8
Ca lactate.....	13.2
K ₂ HPO ₄	22.3
CaH ₄ (PO ₄) ₂	37.0
MgSO ₄ (anhydrous).....	2.4
Mg citrate.....	17.7
Fe citrate.....	6.6

Salt mixture III.

	<i>per cent</i>
KCl.....	10.0
Ca ₂ H ₂ (PO ₄) ₂	33.3
MgSO ₄ (anhydrous).....	6.7
Na ₂ CO ₃ (anhydrous).....	50.0
Fe ₂ O ₃ added.	

Salt mixture II.

	<i>per cent</i>
Ca lactate.....	17.4
MgSO ₄ (anhydrous).....	20.7
K ₂ HPO ₄	48.6
NaCl.....	2.8
Na ₂ SO ₄ (anhydrous).....	10.4
Fe ₂ O ₃ added in small amount.	

Salt mixture IV.

	<i>per cent</i>
Salt mixture I.....	50.0
K citrate.....	50.0

STUDIES OF THE ENDOGENOUS METABOLISM OF THE PIG AS MODIFIED BY VARIOUS FACTORS.

II. THE INFLUENCE OF FAT FEEDING ON ENDOGENOUS NITROGEN METABOLISM.¹

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(Received for publication, September 10, 1913.)

Many investigators have called attention to the fact that feeding fat alone does not spare protein catabolism in the same degree as do carbohydrates.² In fact Cathcart and Landergren have observed that in dogs fat feeding tends to increase nitrogen elimination in a marked degree. The recorded experiments of this character are all of short duration, and no accurate standardization of the lowest possible level of protein metabolism of the experimental animals was made. We deemed it of interest to test the question of the influence of a liberal supply of calories as fat on the endogenous metabolism of pigs which had been carefully reduced to their lowest level of nitrogen output by long continued feeding of a diet of starch, salts and water. The preliminary record would in such experiments serve as a standard for total nitrogen and creatinine elimination, with which the values found under the influence of fat feeding could be compared.

It is difficult to feed a sufficient energy intake in the form of pure fat. Our first attempts, using lard, were not very successful, but butter fat, being much more palatable is taken much more readily by pigs. Since the amounts of fat required in these

¹ Published with the permission of the Director of the Wisconsin Experiment Station.

² Voit: *Handbuch der Physiologie*, Leipzig, 1881, Vol. VI, Part I; Landergren: *Skand. Arch. f. Physiol.*, xiv, p. 112, 1903; Cathcart: *Journ. of Physiol.*, xxxix, p. 311, 1909; Sivén: *Skand. Arch. f. Physiol.*, x, p. 91, 1900; xi, p. 308, 1901.

experiments tend to produce diarrhea we gave the animals paper pulp made from filter paper, with the fat. Salts were given to No. 34 in a mixture nearly neutral (salt mixture II, page 315) and to No. 38 in the form of a decidedly basic mixture (salt mixture III, page 315).

In the case of pig No. 34 (Table I, page 304), 80 grams of butter fat, without any starch, were consumed in the periods following March 25, together with a neutral salt mixture. On the first day there was a marked rise in the nitrogen output, but the average for the period is practically the same as in the preceding starch periods where the same salt mixture was taken. This rise was not accompanied by an increased output of creatinine. During the last period the animal became weakened and there was a decided drop in the output of creatinine.

During the periods of fat feeding with this pig creatine appeared in the urine, and in a few days exceeded in quantity the creatinine nitrogen. Creatine was found in the urine of a number of days in the period when both carbohydrates and fat were fed. The appearance of creatine during exclusive fat feeding is in agreement with the observations made upon other species.³ Acetone and diacetic acid were found only during the last few days of fat feeding.

Pig No. 38 (Table II, page 306) refused on a number of days to eat the full quota of fat. The complication of a deficient energy intake therefore arises, but it is probable that the energy requirement was nearly covered during the entire time. Attention is, however, called to the fact that there was *no sustained rise in the nitrogen output under the influence of butter-fat feeding*. In this connection it might be urged that it is possible that the output of total nitrogen resulting from endogenous metabolism may have fallen to a decidedly lower level than it was in the earlier periods of the experiment. The marked fall in the output of creatinine during the final period (IV) would lend some support to this assumption. If this were the case, the maintenance of the total-N output, under the influence of fat feeding, at nearly the same level as in the earlier periods on starch would in reality be an acceleration of the endogenous metabolism, the total output of nitrogen

³ Mendel and Rose: *this Journal*, x, pp. 213-264, 1911; Myers and Fine: *this Journal*, xv, p. 305, 1913.

being increased without any corresponding increase in the creatinine output. The constancy of the rest nitrogen, and the uniformity in the amounts of ammonia in the third and fourth periods point strongly against such an assumption, and we are of the opinion that there was no sustained stimulation of tissue catabolism as the result of the pure fat diet. It is also of great interest that if creatine was present at all in the urine of No. 38 it was in *very small amounts*, while in the urine of No. 34 large amounts of creatine were present on the same diet. The only difference in the diets of the two pigs was in the character of the salt mixture supplied. With the basic salt mixture creatine was present in *small* amounts or entirely absent, while with the neutral salts the amount was large. We call attention to this point only incidentally at this time since we are making an extended study of the creatine metabolism of the pig in this laboratory. The data available do not warrant conclusions, but the above observation is of interest in connection with the many observations on the conditions under which creatine is eliminated by other animals. The feeding of protein is attended with the production of metabolic acids derived from the sulphur and phosphorus, and of fat by a tendency toward the accumulation of organic acids arising as intermediate products of oxidation. It is possible that this factor is of importance in causing an elimination of creatine.

McCollum and Steenbock⁴ have called attention to the fact that moderate fasting does not lead to the elimination of creatine in the pig as it does in other species and attribute this difference to a greater ability of the pig to utilize fat for energy production. This is supported by the observation that even with high fat feeding acetone and diacetic acid are not eliminated except after a considerable time and then in small amounts only. This idea is also further supported by our further observation that the feeding of butter fat does not cause a permanent rise in the excretion of nitrogen in pigs reduced to their endogenous level through long continued carbohydrate feeding.

⁴ McCollum and Steenbock: this *Journal*, xiii, p. 209, 1912.

SUMMARY OF CONCLUSIONS.

1. Feeding fat as the sole source of energy does not lead to a sustained rise in the nitrogen output of pigs which have been reduced to their lowest possible level of nitrogen metabolism by long continued starch feeding.

2. Fat feeding may produce a considerable elimination of creatine. The total creatinine (creatinine+creatine) may be greatly increased without a corresponding rise in the total nitrogen output.

3. The possibility of the acid or basic character of the ration having an influence on the creatine production is suggested.

STUDIES OF THE ENDOGENOUS METABOLISM OF THE PIG AS MODIFIED BY VARIOUS FACTORS.

III. THE INFLUENCE OF BENZOIC ACID ON THE ENDOGENOUS NITROGEN METABOLISM.¹

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(Received for publication, September 10, 1913.)

The experimental feeding of benzoic acid and benzoates to various animals has been reported by a great number of investigators.² The object of many of these investigations has been to determine whether glycocoll can originate *de novo* in the body for hippuric acid synthesis, or whether hippuric acid can be produced under the influence of benzoic acid or benzoate feeding only in so far as the proteins of the food or the body tissues decompose and yield the glycocoll complex. The conclusions reached as the result of these studies have been various. The more recent papers of Ringer³ and of Epstein and Bookman⁴ summarize the views that have been expressed on this point.

No results have been reported which involve the use of animals reduced to their minimum level of nitrogen output while receiving nitrogen-free food of ample calorific value. A vigorous pig, eating a liberal ration of starch, salts and water, will readily take relatively large doses of benzoic acid for a long period without loss of appetite or signs of illness. It is thus possible to attain complete control of experiments in which the endogenous type of metabolism alone prevails and to obtain ample amounts of urine for the estimation

¹ Published with the permission of the Director of the Wisconsin Experiment Station.

² See Wiechowski: *Beitr. z. chem. Physiol.*, vii, p. 204, 1905-06. All the older literature on hippuric-acid synthesis is referred to and discussed here.

³ Ringer: this *Journal*, x, p. 328, 1911.

⁴ Epstein and Bookman: this *Journal*, x, p. 353, 1911.

of all the determinable nitrogen compounds eliminated, and at the same time to continue the experiments long enough to make the results conclusive. Relatively long preliminary periods on a starch diet serve to standardize the experimental animal in a manner more satisfactory than can be attained with any species which has been employed hitherto.

Although no complete resumé of the literature of the subject is desirable here it may be well to refer to a few of the experiments most closely related to our own, which will serve to show the lack of unanimity of opinions arrived at by others. Parker and Lusk,⁵ employing rabbits, reached the conclusion that only 3-5 per cent of the total nitrogen of starvation can appear as hippuric acid when benzoic acid is fed, which represents an amount of glycocoll which would be yielded by the proteins of the body on acid hydrolysis. Wiechowski,⁶ employing guinea pigs, found that moderate doses of benzoic acid do not lead to an increased nitrogen elimination and observed the ratio of hippuric acid N to total N to be as high as 64 : 100. He believes that glycocoll can be synthesized at the expense of urea. Magnus-Levy⁷ criticized the method of calculation of Wiechowski, and holds that his values are excessive, but is essentially in accord with the latter in regarding the glycocoll content of the metabolized tissues insufficient to account for the hippuric acid eliminated.

Ringer⁸ has recently employed both rabbits and goats and concluded that the ingestion of benzoic acid caused an increased nitrogen elimination, and that from the nitrogen of this "extra destroyed protein," is derived the glycocoll for hippuric acid synthesis. Urea nitrogen he found not materially affected, but observed as high as 36 per cent of the total nitrogen appearing as hippuric acid. Ringer holds that the glycocoll resulted not from a deviation of the course of normal intermediary metabolism, but had its origin rather in a specific and peculiar metabolic process.

Epstein and Bookman,⁹ employing rabbits, concluded that benzoic acid exerts a truly toxic effect, but acts in a selective way, causing the elimination of excessive amounts of nitrogen which is almost entirely accounted for in the hippuric acid eliminated. It is apparent therefore that a clarification of existing views is highly desirable.

We were led to believe from the recorded data, that the ingestion of benzoic acid would stimulate endogenous metabolism, when this type of protein catabolism alone prevailed. That this is not the

⁵ Parker and Lusk: *Amer. Journ. of Physiol.*, iii, p. 472, 1900.

⁶ Wiechowski: *loc. cit.*

⁷ Magnus-Levy: *Biochem. Zeitschr.*, vi, p. 521, 1907.

⁸ Ringer: *loc. cit.*

⁹ Epstein and Bookman: *loc. cit.*

case is made apparent by an inspection of Tables IV and V, pages 310 and 313. The experimental procedure was the same as that employed in the experiments described in the two preceding papers. The pigs were reduced to their lowest level of nitrogen elimination by a preliminary starch period, so that the factors of energy intake and exogenous protein metabolism are eliminated. The acid or basic character of the ration has been varied under controlled conditions. The experiments are believed to be of sufficient duration to give reliable data concerning the points under consideration.

During the first period of twelve days pig No. 39 (Table IV, p. 310) received a ration of starch plus alkaline salts (salt mixture IV). Having obtained the necessary preliminary data, 4 grams of benzoic acid per day were added to the ration, in two feeds. No marked rise in the total nitrogen output was observed. The urea N decreased, while the ammonia N remained constant.

On April 26 the daily dose of benzoic acid was increased to 10 grams. There occurred a sudden rise in all the nitrogenous constituents of the urine. The total N rose to more than double that of the preceding day. The creatinine, though increased, was not in proportion to the total. On the following day the total nitrogen sank again, and the average for the rest of the period is slightly less than for the preliminary period. Of special interest is the average daily elimination of urea N. This decreased to less than one-half the quantity excreted in the previous periods. The decrease is in fact great enough to account for all the nitrogen necessary for the synthesis of hippuric acid equivalent to the benzoic acid fed. During the following period the dose of benzoic acid was increased to 16 grams per day. A rise in the total N output followed, while the urea N remained practically constant at its minimum level.

In the final period the salt mixture was changed from an alkaline to a neutral one and 10 cc. of 1 : 4 HCl were added in addition to the benzoic acid, as a part of the work discussed in the first paper of this series (p. 299). It was thought of interest to observe the effect of superimposing upon the endogenous metabolism, a demand for nitrogen, both for neutralization of the acid, and for hippuric acid synthesis. The result was a marked rise in total-N and ammonia-N output. The previous low figure for urea N remained practically unaltered.

The work with pig No. 39 was duplicated with pig No. 43 and the data are presented in Table V (page 313). The data obtained with this pig confirm those of the former animal in all respects. Under the influence of benzoic acid the total nitrogen output was not increased unless the dose was very large, and then the rise was only temporary. The urea nitrogen drops at once and hippuric acid is without doubt produced from the nitrogen which would have appeared in this fraction had no benzoic acid been given. The ammonia nitrogen is not changed in amount by the presence of benzoic acid in the diet. In the case of pig No. 43, weighing only 49 pounds, the daily dose of 10 grams of benzoic acid is relatively high and it seems improbable that larger amounts would lead to data of greater significance than are afforded by these experiments. In this table as in table IV it is apparent that the temporary rise in the nitrogen output at the beginning of the benzoic acid feeding affects the creatinine output but little if at all.

From a consideration of these results it seems fair to conclude that in the body of the pig, a considerable portion of the nitrogen which, in the normal metabolic processes would be converted into urea, may be diverted to the synthesis of hippuric acid. Excessive doses of benzoic acid will not, however, serve to reduce the urea-N fraction below a certain constant level, viz., about 20 per cent of the total. An increase in the total nitrogen output will occur instead. This view is essentially in harmony with that of Wiechowski, and is at variance with those of Ringer and of Epstein and Bookman. It seems probable that the discrepancies may find an explanation in the very short period of observation made by these investigators. In experiments of very short duration, complicated by the presence of exogenous protein catabolism, excessive doses of benzoic acid, causing a sudden increase in the output of total nitrogen may entirely obscure the relation between hippuric acid and urea. It is of course possible that different species of animals may react differently under these experimental conditions.

SUMMARY OF CONCLUSIONS.

1. A considerable amount of the nitrogen which appears in the form of urea in pigs reduced to the endogenous level of protein metabolism, may be converted into glycocoll when benzoic acid is fed, for the purpose of hippuric acid synthesis.

2. When the quantity of benzoic acid ingested is not excessive, there is no noticeable rise in the total nitrogen excreted, over that which is eliminated on the same diet without benzoic acid.

3. When the quantity of benzoic acid ingested is very large, there is a marked increase in the output of total nitrogen catabolized. The urea nitrogen cannot be reduced to a lower level than about 20 per cent of the total.

4. No change in the creatinine output is observed when the protein catabolism is stimulated by excessive doses of benzoic acid.

5. Endogenous protein metabolism appears to consist of at least two types. One can be stimulated greatly for ammonia production by the introduction of mineral acids, or for hippuric acid when benzoic acid is introduced; the other, measured by creatinine, remains unaffected by the methods we have described.

THE NON-INTERFERENCE OF "PTOMAINES" WITH CERTAIN TESTS FOR MORPHINE.

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(Received for publication, September 26, 1913.)

In trials for murder by morphine poisoning the defense often insists that the reactions obtained have been, or may have been, caused by ptomaines and not by morphine. It has often been stated that many tests for alkaloidal poisons may be simulated so closely by various bacterial products formed during the putrefaction of biological material that it becomes impossible to distinguish between them.¹

Vaughan² obtained by anaerobic putrefaction of certain tissues, a substance which he thought gave certain reactions similar to those due to morphine and claimed "*that the tests for morphine by following the scheme of Dragendorff are altogether untrustworthy.*" Witthaus,³ however, claims in contradiction to Vaughan *that if a residue obtained by a properly conducted Dragendorff or other appropriate method gives the following reactions, ferric chloride, Fröhde's, Pellagri's, Husemann's, nitric acid, and iodic acid, with distinctness and purity, the proof of the presence of morphine is quite as complete as it is in the case of nitric acid, whose presence is unhesitatingly assented by chemists upon the evidence of color reactions.*

We would like to state that on starting the work to be described in this paper we felt that Vaughan's idea was correct, and this

¹ It has been claimed that bacterial products may give reactions similar to those due to morphine, conine, nicotine, atropine, strychnine, digitalin, veratrine, colchicine, and delphinine.

² *Trans. Assoc. Amer. Phys.*, ix, p. 249, 1894; Peterson and Haines: *Text-book of Legal Med. and Toxicology*, 1904, 2, p. 690.

³ Witthaus and Becker: *Med. Jurisprudence, Forensic Med. and Toxicology*, 1911, iv, p. 999.

work was commenced with the hope of being able to devise some method to distinguish the reactions of morphine from putrefactive products. However, we found no difficulty in distinguishing the reactions due to morphine from those due to putrefactive products and agree therefore with the claim of Witthaus stated above.

Methods. About 5 kgm. of a mixture of human liver, pancreas, kidney, intestines, stomach, brain and heart muscle were chopped finely and divided into two portions. One portion was placed in a wide mouthed jar, exposed to the air and allowed to putrefy for fifty days (Solution A). The other portion was placed in a large bottle and securely stoppered with a perforated cork connected with a bent glass tube. The cork was sealed with paraffin and the outer end of the glass tube was allowed to dip into a cistern of mercury thus excluding all communication with the outside air. This tissue in the bottle was allowed to stand for fifty days (Solution B). At the end of this time the contents of the two bottles were poured into separate dishes.

It may be seen that the bottle "Solution A" contained the products of aerobic putrefaction of certain human organs while "Solution B" contained the products of anaerobic putrefaction of the same organs. Solutions A and B were then divided into four portions and to two portions of each solution 150 mgm. of morphine sulphate were added. One portion of Solution A (aerobic putrefaction) without addition of morphine sulphate and one portion with addition of morphine sulphate were then subjected to the Stas-Otto method of extraction. Similar portions of Solution A were also subjected to the Dragendorff process. One portion of Solution B (anaerobic putrefaction) without addition of morphine sulphate and one portion with addition of morphine sulphate were subjected to the Stas-Otto method. Similar portions of Solution B were also subjected to the Dragendorff process.⁴

The following tabulated summary contains the results obtained in this study, *showing that bacterial products formed during aerobic and anaerobic putrefaction of certain human organs did not in any way give reactions simulating those due to the presence of morphine*

⁴The Stas-Otto method was carried out as described by Autenrieth: *The Detection of Poisons and Strong Drugs*, 1909; the Dragendorff method was carried out as described by Witthaus (*loc. cit.*, p. 157).

and in no way interfered with the detection of morphine, when morphine was added to these putrefactive products.

In the following tables + means that a typical reaction was obtained, while - means that no color at all was produced by the reagent. In those cases where the color is mentioned, it indicates that while the reaction was negative still a color was produced by the reagent. The various tests mentioned for morphine were carried out according to the directions given by Witthaus.

TABLE I.
Results of Stas-Otto method of extraction.

EXTRACTS	No morphine sulphate added to these solutions.													
	SOLUTION A (AEROBIC)							SOLUTION B (ANAEROBIC)						
	TESTS							TESTS						
	Nitric Acid	Husemann's Pellagris'	Fröhde's	Marquis'	Iodic acid	Ferric chloride	Prussian blue	Nitric Acid	Husemann's Pellagris'	Fröhde's	Marquis'	Iodic acid	Ferric chloride	Prussian blue
Ether extract of acid solution.....	pale pink	-	dirty brown	-	-	dirty gray	+	pale pink	-	-	-	-	gray	+
Ether extract of sodium hydroxide solution.....	-	-	pink	-	-	-	+	-	-	-	-	-	-	+
Ether extract of ammoniacal solution.	-	-	dirty brown	-	-	-	+	-	-	-	-	-	-	+
Chloroform extract of ammoniacal sol.	-	-	-	-	very pale pink	-	+	-	-	-	-	pale pink	-	+
150 mgm. of morphine sulphate added														
Ether extract of acid solution.....	pale pink	-	brown	-	-	-	+	pale pink	-	-	-	-	-	+
Ether extract of sodium hydroxide solution.....	-	-	brown	-	-	-	+	-	-	-	-	-	-	+
Ether extract of ammoniacal solution	+	++	+	+	+	?	+	+	++	++	+	+	?	+
Chloroform extract of ammoniacal sol.	+	++	+	+	+	+	+	+	++	++	+	+	+	+

Tests for Morphine

TABLE II.

*Results of Dragendorff extraction.**

EXTRACTS	No morphine sulphate added to these solutions												
	SOLUTION A (AEROBIC)						SOLUTION B (ANAEROBIC)						
	TESTS						TESTS						
	Nitric acid	Husemann's Pellagris'	Fröhde's	Marquis'	Iodic acid	Ferric chloride Prussian blue	Nitric acid	Husemann's	Pellagris'	Fröhde's	Marquis'	Iodic acid	Ferric chloride
Petroleum ether ext. of acid sol.	-	-	-	-	-	-	-	-	-	-	-	-	-
Benzene extract of acid solution	-	-	-	-	-	-	-	-	-	-	-	-	+
Chloroform ext. of acid sol.	pink	-	-	-	-	+	pink	-	-	-	-	-	+
Ether extract of acid sol.	pink	-	-	-	-	+	pink	-	-	-	-	-	+
Petrol. ether ext. of ammon. sol.	-	-	-	-	-	-	-	-	-	-	-	-	+
Benzene extract of ammoniacal sol.	-	-	-	-	-	-	-	-	-	-	-	-	-
Chloroform ext. of ammoniacal sol.	-	-	-	-	-	-	-	-	-	-	-	-	-
Amyl alcohol ext. of ammon. sol.	-	-	-	-	-	-	brown yellow	-	pink	-	-	violet	green
150 mgm. of morphine sulphate added						150 mgm. of morphine sulphate added							
Petroleum ether ext. of acid sol.	-	-	-	-	-	-	-	-	-	-	-	-	-
Benzene extract of acid solution	-	-	-	-	-	-	-	-	-	-	-	-	+
Chloroform ext. of acid solution	pink	-	-	-	-	+	-	-	-	-	-	-	+
Ether extract of acid solution	pink	-	-	-	-	+	-	-	-	-	-	-	+
Petrol. ether ext. of ammon. sol.	-	-	-	-	-	-	-	-	-	-	-	-	+
Benzene ext. of ammon. sol.	-	-	-	-	-	-	-	-	-	-	-	-	-
Chloroform ext. of ammoniacal sol.	+	+	+	+	+	+	+	+	+	+	+	+	+
Amyl alcohol ext. of ammon. sol.	+	+	+	+	+	+	+	+	+	+	+	+	+

* These results also show the absolute worthlessness of the Prussian blue test.

BACTERIAL AND ENZYMIC CHANGES IN MILK AND CREAM AT 0°C.¹

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INTRODUCTION.

Previous work² in this laboratory has demonstrated that raw milk, held at or a little below a temperature of 0°C., undergoes a marked proteolysis, which is pronounced at the end of two weeks. One function of the present research was to determine what part of this proteolysis must be ascribed to the native enzymes of the milk and what part to the bacterial flora, and, finally, to determine the combined action of these two agents. The action of the milk

¹ This paper was presented at the Third International Congress of Refrigeration, Chicago, 1913. It is the report of work done during the winter of 1908-09, when the laboratory was confronted with the task of studying the effect of low temperatures on flesh foods. There were no precedents or methods to guide us, and the apprehensions of the public demanded that results be obtained promptly lest the public health suffer. We, therefore, made a series of observations on the chemical and bacteriological changes occurring in milk and cream, to determine as quickly as possible the general trend of the decomposition at low temperatures, and conducted our work on flesh changes accordingly. Part of the preliminary work has already been published in this *Journal*; the remainder constitutes the present communication, issued in the hope that the fundamental facts which developed as the work progressed, may be of service to others as they were to us. It would have been impossible to execute such a study promptly had there not been unusually efficient team work on the part of the investigators, and an intelligent cross interest of chemists and bacteriologists in the entire scope of the work. Dr. J. S. Hepburn, in addition to his share of the chemical analyses, has correlated and presented the data, and to him the thanks of all the authors are due.—M. E. PENNINGTON.

² This *Journal*, iv, p. 353, 1908.

enzymes took place in formolized raw milk, of the bacterial flora in reinfected sterile milk, and of the two agents in combination in raw untreated milk. Sterile milk was also studied as a control.

Since in the previous studies, it was shown that the acidity of raw milk increases³ and that the lactose content decreases,⁴ when raw milk is held at 0°C., it was considered advisable to include within the scope of the present investigation, determinations of acidity and lactose, and to line up the chemical changes with the changes in the freezing point. At the same time, a similar set of experiments was carried out on cream, the chemical analysis including the various fat constants, the lecithin and the freezing point. Certain zymochemical investigations were made on both the milk and the cream. The research cited showed that bacterial growth to a high degree had taken place in the milk during its progress,⁵ so the number, groups and species of organisms, present in the various samples of milk and cream, were determined. The bacterial work possessed an added interest, inasmuch as it rendered possible a comparison of the relative rate of growth of the milk organisms in reinfected sterilized clean milk (or cream) and raw untreated clean milk (or cream) at a temperature of 0°C.

METHODS.

Preparation of the samples.

The raw milk was fresh clean milk from a high-grade dairy and was strictly comparable with the milk certified by medical milk commissions. The portion for the study of sterile and reinfected sterile milk was received at the laboratory two days in advance of the remainder of the sample, and was sterilized by heating in an Arnold steam sterilizer for thirty minutes on each of three successive days. The raw untreated milk was stored without treatment of any kind. The formolized milk was prepared by adding sufficient formaldehyde to raw clean milk to make 0.1 per cent. This is the quantity of formaldehyde that was used by Tice and Sherman⁶ in their study of proteolysis of milk at the temperature of the laboratory.

The reinfected milk was prepared by the method of St. John and Pennington.⁷ The organisms were precipitated from a portion of the raw milk by

³ Pennington: *loc. cit.*

⁴ Hepburn: *Journal of the Franklin Institute*, clxxii, p. 187, 1911.

⁵ Pennington: *loc. cit.*

⁶ Tice and Sherman: *Journ. of the Amer. Chem. Soc.*, xxviii, p. 189, 1906.

⁷ St. John and Pennington: *Journ. of Inf. Dis.*, iv, p. 647, 1907.

centrifugalization at high speed in sterile glass tubes, which have the general shape of tubes for the centrifugal collection of urinary sediment, but have a capacity of 250 cc. The centrifuge carried eight such tubes and was run at a velocity of 3,000 revolutions per minute. A picture of the apparatus has been published.⁸ The supernatant milk was removed by means of sterile pipettes; sterile physiological salt solution was added and mixed with the bacterial sediment; then the tubes were returned to the centrifuge and whirled. The supernatant solution was removed from the bacterial sediment with sterile pipettes, and the entire procedure was repeated several times in order to obtain organisms as free as possible from milk serum. The organisms were then sown in a portion of the sterile milk at the temperature of the room; care was taken that the total count per cubic centimeter of the reinfected milk should be approximately the same as the total count per cc. of the raw untreated milk.

Each of the four samples—raw untreated, formolized raw, reinfected sterile and sterilized—was kept in a sterile flask in a mechanically refrigerated chill-room at 0°C. At intervals of one week, the contents of each flask were mixed intimately by thorough shaking, and a sample was withdrawn by means of a large sterile pipette, placed in a sterile flask and submitted to analysis—bacterial, zymochemical and chemical. The sterile sample was analyzed at the beginning and at the end of the experiment. Its sterility was demonstrated at both times by plating. Needless to remark, on the first analysis of the fresh samples, one analysis sufficed for both the raw untreated and the formolized raw milk, and one analysis for both the sterilized and the reinfected sterile milk.

The source, preparation and sampling of the cream were the same as in the case of the milk.

The milk was held at 0°C. for a maximum period of thirty-five days, the cream for a maximum period of twenty-eight days.

Bacterial and zymochemical studies were made on both milk and cream.

For the chemical analysis, the milk served for a study of changes in freezing point, lactose, acidity and distribution of the nitrogen, while the cream served for a study of the freezing point and of the lipins, including the determination of the various fat constants and of the quantity of lecithin.

Chemical technique.

The total nitrogen, casein nitrogen, and albumin and syntonin nitrogen, were determined by the method of Van Slyke and Hart⁹ as modified in this laboratory.¹⁰ The caseose nitrogen was determined in the filtrate from the

⁸ Hepburn: *Journal of the Franklin Institute*, clxxi, p. 595, 1911.

⁹ Van Slyke and Hart: New York Agricultural Experiment Station, Geneva. Bulletin 215, 1902, p. 101.

¹⁰ Pennington: *This Journal*, iv, p. 360, 1908; Hepburn: *Journal of the Franklin Institute*, clxxii, p. 390, 1911.

albumin and syntonin by the method of Bömer¹¹ for proteose nitrogen. The amino-acid nitrogen was determined on a separate portion of the milk by the method of Bigelow and Cook.¹² In all cases the actual determination of the nitrogen in its various forms was by the Gunning method.

The determination of total nitrogen including nitrate nitrogen, was carried out according to the modified Gunning method¹³ of the Association of Official Agricultural Chemists, using salicylic acid and sodium thiosulphate.

The free ammoniacal nitrogen was determined according to Berg and Sherman.¹⁴

In the tabulated results (Table I) the data included as undetermined nitrogen may be taken as a measure of peptone nitrogen.

The percentage of each form of nitrogen in terms of the milk is given in the tables in Roman type, while each form is also expressed as per cent of the total nitrogen of the milk by means of italics. The latter mode of expression of nitrogen results is of great help in the study of proteolysis.

Acidity was determined by titrating 10 cc. of milk at room temperature with $\frac{N}{10}$ sodium hydroxide, using phenolphthalein as the indicator. The results are expressed as cc. of $\frac{N}{10}$ sodium hydroxide required by 100 cc. of milk.

The lactose was determined by the optical method, after clarification by means of acid mercuric nitrate.¹⁵

The lecithin was extracted as directed by Nerking and Haensel¹⁶ and was burned by means of sodium peroxide, as described by Le Clerc and Dubois¹⁷ and by Dubois.¹⁸ The lecithin phosphoric anhydride was then determined volumetrically by solution of the ammonium phosphomolybdate in a known volume of standard alkali, and titration of the excess of alkali. The per cent of lecithin was calculated by multiplying the per cent of lecithin phosphoric anhydride by the factor 11.41.

The fat for the determination of fat constants was extracted from the cream by the method used in this laboratory for the extraction of fat from egg yolk.¹⁹ The cream was mixed with several times its volume of 95 per cent alcohol, and the precipitate was collected on a filter and dried over cal-

¹¹ Bömer: *Zeitschr. f. anal. Chem.*, xxxiv, p. 562, 1895.

¹² Bigelow and Cook: *Journ. of the Amer. Chem. Soc.*, xxviii, p. 1485, 1906.

¹³ U. S. Department of Agriculture, Bureau of Chemistry, Bulletin 107, revised, p. 8.

¹⁴ Berg and Sherman: *Journ. of the Amer. Chem. Soc.*, xxvii, p. 124, 1905.

¹⁵ U. S. Department of Agriculture, Bureau of Chemistry, Bulletin 107, revised, p. 118.

¹⁶ Nerking and Haensel: *Biochem. Zeitschr.*, xiii, p. 348, 1908.

¹⁷ Le Clerc and Dubois: *Journ. of the Amer. Chem. Soc.*, xxvi, p. 1108, 1904.

¹⁸ Dubois: *ibid.*, xxvii, p. 729, 1905.

¹⁹ Pennington: *this Journal*, vii, p. 115, 1910.

cium chloride in a desiccator. The filtrate was evaporated to dryness *in vacuo*, using a water bath as a source of heat; and the residue was combined with the precipitate, then extracted for two days in a Soxhlet extractor with freshly distilled petroleum ether of boiling point 40–60°C. The solvent was removed by distillation on the water bath, and the residue of butter fat was used for the study of the fat constants, which were determined by the methods of the Association of Official Agricultural Chemists.²⁰ The procedure of Hanus was used for the determination of the iodine number, while that of Leffmann and Beam served for the determination of the Reichert-Meissl number. The index of refraction was taken on an Abbe refractometer which was provided with a water jacket. The ester value was determined by difference by subtracting the acid value from the saponification number.

For the determination of the freezing point, the apparatus of Beckmann was used. The fixed point of the Beckmann thermometer was adjusted and determined by means of dimethylaniline.

Zychochemical methods.

For the detection of catalase 5 cc. of milk or cream and 0.3 cc. of $\frac{M}{10}$ hydrogen peroxide were mixed in a sterile Erlenmeyer flask of 50 cc. capacity, provided with a sterile delivery tube which dipped beneath a eudiometer in a pneumatic trough. The oxygen evolved was thus collected over water by upward, wet displacement. The period of incubation was forty-eight hours at 37.5°C. The volume of oxygen was measured at room temperature, which was noted as well as the barometric pressure. The difference of level of the water within and without the eudiometer was disregarded. The volume of oxygen has been reduced to a temperature of 0°C. and to a pressure of 760 mm. of mercury for insertion in the tabulated results.

In the study of reductases, the reagents—methylene blue and methylene-blue-formaldehyde—were prepared and used as directed by Schardinger.²¹ No attempt was made to obtain anaerobic conditions; the period of incubation was from twelve to twenty-four hours at 37.5°C. A bleaching of the lower portion of the solution during that period of time was considered proof of the presence of reductase, even though a blue ring remained on the surface of the substratum.

The oxidase reagents were tincture of guaiac U.S.P.²² and trikresol—a 3 per cent aqueous solution. Ten cubic centimeters of milk and 1 cc. of the reagent were mixed and incubated at 37.5°C. for twelve to twenty-four

²⁰ U. S. Dept. of Agric., Bureau of Chemistry, Bulletin 107, revised, pp. 131–142.

²¹ Schardinger: *Zeitschr. f. Untersuchung der Nahrungs und Genussmittel*, v, p. 1113, 1902.

²² *Pharmacopoeia of the United States of America*, 8th Decennial Revision, p. 467.

hours. The production of a blue color by the guaiac and of a violet or purple color by the trikresol during the period of incubation was considered to indicate the presence of an oxidase.

In the tabulated results, the presence of either of the reductases or of either of the oxidases is designated by a plus sign (+) in the proper column, while the absence of the enzyme is recorded by a minus sign (-) in the proper column.

Bacteriological technique.

To determine the total number of organisms per cc., plain nutrient agar was sown with the milk or cream.

To determine the number of acid-formers present, plates of litmus lactose agar were sown and a count was made of all acid-forming colonies.

To determine the number of anaerobes and facultatives present, plates of plain nutrient agar were sown according to Wright's anaerobic plate method.

In every case the dilutions were carried high enough to insure between one hundred and two hundred organisms on each plate, and duplicate plates were always made of every dilution used.

The plates were incubated at 37°C. for two days, 20°C. for five days, and 0°C. for four weeks. A Stewart counting chamber and a 1½ inch lens were used for counting; the results were recorded as directed by the American Public Health Association.²³

To estimate the number of liquefying organisms present, sowings were made in nutrient gelatin; the plates were incubated at 20°C. for five days and the number of organisms liquefying the medium were counted and the results recorded as stated above.

Isolation and identification of organisms.

For the isolation, identification and comparative rate of growth of the organisms in the milk and the cream, plates were selected where the colonies had sufficient space for free development. All the colonies were counted, and those with like cultural characteristics were grouped and studied as to their morphology and relative rate of growth and appearance of the agar streak, when incubated at 37°C. (incubator), 22°C. (room), and 20°C. (refrigerator), respectively. The optimum temperature for each organism isolated was used for the further study of its morphology and biochemical characteristics. Characteristic growth on plates of plain nutrient agar, litmus lactose agar, and nutrient gelatin, respectively, were studied, also growth in stab culture on nutrient agar and nutrient gelatin. The characteristic growth and reaction produced by the organisms in neutral bouillon and in litmus milk were noted, as were indol production, reduction

²³ American Public Health Association: *Standard Methods for the Examination of Water and Sewage*, 2nd edition, 1912, p. 79.

of nitrate to nitrite, digestion of casein and of gelatin, chromogenicity and fluorescence. The aerobic, anaerobic and facultative properties were studied as well as morphology, motility, arrangement of flagella, spore-formation and reaction toward the Gram stain.

THE CHEMICAL CHANGES IN THE MILK DURING HOLDING.

The distribution of the nitrogen.

On the first analysis of the reinfected sterile and the sterilized milks, it was seen that the heat during sterilization had given rise to a partial coagulation of the albumin; this coagulated albumin precipitated with the casein in the analytic separation of the two proteins, hence the casein nitrogen was high and the albumin and syntonin nitrogen low for a fresh milk. The milk had also been slightly concentrated by sterilization as shown by the total nitrogen which was slightly higher in the sterile reinfected and the sterilized milks than in the raw untreated and the formolized raw milks.

In the *raw, untreated* milk, the casein nitrogen underwent a progressive decrease; the albumin and syntonin nitrogen was almost the same at the beginning and end of the experiment, its fluctuations during the intermediate analyses being doubtless due to formation and decomposition of metaprotein at the expense of the casein. The digestion of the casein must account for the large increase in caseose nitrogen during the latter half of the period of holding. That the caseoses may undergo digestion is shown by the progressive decrease in that form of nitrogen during the first half of the experiment. The amino-acid nitrogen showed a marked tendency to increase at the expense of the protein nitrogen. During the proteolysis, peptones were doubtless formed as is shown by the variations in the data listed as "undetermined nitrogen,"²⁴ which is a measure of peptone nitrogen by difference. The quantity of free ammoniacal nitrogen was but slight and fluctuated wildly. Nitrates were not formed during holding, for, at the end of the experiment, the total nitrogen and total nitrogen including nitrates were practically the same, hence, nitrogen fixers were absent from the milk.

Ravenel, Hastings and Hammer²⁴ analyzed a clean milk and a fair grade of commercial milk which had been held at low tempera-

²⁴ Ravenel, Hastings and Hammer: *Journ. of Inf. Dis.*, vii, p. 38, 1910.

tures for a period of 203 days. In addition to the total nitrogen, they record the "water-soluble" nitrogen which was obtained by diluting the milk with water, adding "a small amount" of acetic acid at the temperature of the water bath, filtering and determining the nitrogen content of the filtrate. During holding, the soluble nitrogen—expressed as per cent of the total nitrogen—became higher than in fresh milk, being 17.97 in the clean milk and 22.38 in the commercial milk kept at $-9^{\circ}\text{C}.$; and over 72 in both milks kept at $0^{\circ}\text{C}.$ In the latter experiments the total nitrogen decreased to a marked degree; the loss is ascribed to the liberation of elementary nitrogen. These investigators refer the proteolysis at $0^{\circ}\text{C}.$ to bacterial action; that at $-9^{\circ}\text{C}.$ to the action of the native milk enzyme galactase. Since their method of chemical analysis differed widely from that used in the present research the results obtained in the two studies are not strictly comparable.

In the *reinfected, sterile* milk, the casein nitrogen decreased progressively but to a far less degree than did the casein nitrogen of the raw untreated milk. The albumin and syntonin nitrogen varied within narrow limits and showed no marked change during the period of keeping. The caseose nitrogen showed a progressive increase most marked during the last third of the experiment. The amino-acid nitrogen increased to some extent, and the peptone nitrogen tended to decrease. Since, on the final analysis, the total nitrogen and the total nitrogen including nitrates were the same, nitrates were not formed during holding. Hence, nitrogen fixers were not present in the milk. The amount of free ammoniacal nitrogen was small and apparently tended to decrease.

In the *formolized, raw* milk, the casein nitrogen remained practically constant during the entire period of holding. The albumin and syntonin nitrogen tended to decrease and the caseose nitrogen to increase. The peptone nitrogen showed a tendency to decrease, and the amino-acid nitrogen a tendency to increase. The free ammoniacal nitrogen was a negligible quantity. In this connection it should be mentioned that Tice and Sherman,²⁵ during a study of formolized raw milk held at room temperature for periods as long as thirty-seven months, noted that "the albumin was largely digested before the original amount of casein was appreciably reduced." Hence, at the temperature of the chill-room and of the

²⁵ *Loc. cit.*

room, the same type of proteolysis occurs in formolized raw milk, and is produced mainly, if not entirely, by galactase—a native milk enzyme; possibly proteolytic enzymes derived from the dead bacteria, may also participate in the digestion of the protein. On the other hand, Sherman, Berg, Cohen and Whitman²⁶ reported that the free ammoniacal nitrogen increased in formolized raw milk kept in the room at 15°C. for three months, while in the present research the free ammoniacal nitrogen was found to be an absolutely negligible quantity in formolized raw milk held at 0°C.

In the *sterilized* milk, during the entire period of storage, the changes in the distribution of the nitrogen were but slight, and, on the whole, lie within the limits of analytic error.

The study of the nitrogen results leads to the following conclusions. The proteolysis of the casein is, primarily, of bacterial origin, since it occurred in the reinfected sterile milk, but not in the formolized raw milk. The digestion of the albumin and syn-tonin is, primarily, due to native enzymes of the milk, since it took place in the formolized raw but not in the reinfected sterile milk. In raw untreated milk, however, the native enzymes and bacterial flora act in combination in giving rise to more rapid proteolytic changes, since in the same period of time—five weeks—over twice as much casein was digested in the raw untreated milk as in the reinfected sterile milk. The general trend of the proteolysis, enzymic, bacterial and combined, is toward a tryptic digestion, that is, the passage through caseose and peptone to amino-acids which accumulate as the period of holding lengthens. The changes in the ammoniacal nitrogen are negligible.

Acidity.

The acidity of the *raw, untreated* milk increased more or less progressively to the highest values of the entire series of experiments. In the *reinfected, sterile* milk, the acidity increased progressively and finally attained values which were second only to those obtained in the raw untreated milk. In the *formolized, raw* milk the acidity first increased, then decreased to a value which remained fairly constant to the very end of the experiment. The initial rise was possibly due to the bacterial enzymes of the dead

²⁶ Sherman, Berg, Cohen and Whitman: *This Journal*, iii, p. 171, 1907.

TABLE I.

Chemical changes in raw and treated clean milk kept at 0° C.

(The per cent of the various forms of nitrogen in the milk are printed in Roman; the various forms of nitrogen are also printed as per cents of the total nitrogen in italics. Lactose is expressed as per cent and acidity as cc. of $\frac{N}{10}$ NaOH required to neutralize 100 cc. of milk.)

PERIOD OF HOLD- ING AT 0°C.	DISTRIBUTION OF THE NITROGEN							LACTOSE	ACIDITY	FREEZING POINT
	Total N*	Casein N	Albumin and syntonin N	Caseose N	Amino-acid N	Undeter- min- ed N	Free aminon- iacal N			
<i>Raw untreated milk.</i>										
<i>days</i>										<i>deg. C.</i>
Fresh	0.585	0.457	0.067	0.033	0.007	0.019	0.00047	5.3	18.0	-0.550
		78.12	11.79	5.64	1.20	3.25	0.080			
7	0.571	0.432	0.084	0.018	0.018	0.019	0.00046	5.2	16.5	-0.540
		75.66	14.71	3.15	3.15	3.33	0.080			
14	0.566	0.377	0.081	0.000	0.032	0.076	0.00000	3.8	32.7	-0.565
		66.61	14.31	0.00	5.65	13.43	0.000			
21	0.569	0.335	0.069	0.167	0.033	0.035	0.00075	3.7	30.8	-0.595
		58.88	12.13	29.35	5.80	6.15	0.131			
28	0.537	0.295	0.053	0.161	0.022	0.006	0.00113	3.3	46.5	-0.585
		54.93	9.87	29.98	4.10	1.12	0.210			
35	0.572	0.286	0.066	0.136	0.044	0.040	0.00040	3.5	56.0	-0.630
		50.00	11.54	23.78	7.69	6.99	0.069			
<i>Reinfected sterile milk.</i>										
Fresh	0.608	0.528	0.018	0.029	0.010	0.023	0.0029	4.5	25.8	-0.735
		86.84	2.96	4.77	1.64	3.78	0.477			
7	0.595	0.516	0.021	0.016	0.030	0.012	0.00356	5.1	26.0	-0.550
		86.72	3.53	2.69	5.04	2.02	0.598			
14	0.585	0.522	0.015	0.029	0.033	0.014	0.00410	4.2	32.0	-0.560
		89.23	2.56	4.96	5.64	2.39	0.701			
21	0.590	0.507	0.013		0.027		0.00210	4.4	30.8	-0.585
		85.93	2.20		4.58		0.356			
28	0.592	0.463	0.015	0.080	0.014	0.020	0.00168	3.3	41.3	-0.570
		78.21	2.53	13.51	2.36	3.38	0.284			
35	0.596	0.444	0.023	0.105	0.026	0.002	0.00108	3.5	51.8	-0.680
		74.50	3.86	17.62	4.36	0.34	0.181			

*Total nitrogen, including nitrate nitrogen, at 35th day of holding: raw untreated milk, 0.565, reinfected sterile milk, 0.595.

†In this set of analyses, the sum of the nitrogenous constituents determined exceeds the total nitrogen of the milk by this amount.

TABLE I—Continued.

PERIOD OF HOLD- ING AT 0°C.	DISTRIBUTION OF THE NITROGEN							LACTOSE	ACIDITY	FREEZING POINT
	Total N	Casein N	Albumin and syntonin N	Casoose N	Amino-acid N	Undeterm- ed N	Free ammon- iacal N			
<i>Formolized raw milk.</i>										
<i>days</i>										<i>deg. C.</i>
Fresh	0.585	0.457	0.069	0.033	0.007	0.019	0.00047	4.8	18.0	-0.550
	78.12	11.79	5.64	1.20	3.25	0.080				
7	0.573	0.450	0.056	0.023	0.015	0.029	0.00060	5.1	19.0	-0.660
	78.53	9.77	4.01	2.62	5.06	0.010				
14	0.564	0.463	0.044	0.031	0.026	0.000	0.00000	4.4	27.8	-0.650
	82.09	7.80	5.50	4.61	0.00	0.000				
21	0.550	0.465	0.051		0.025		0.00000	5.0	19.8	-0.655
	84.55	9.27			4.55		0.000			
28	0.561	0.465	0.032	0.048	0.012	0.004	0.00000	4.8	21.0	-0.655
	82.89	5.70	8.56	2.14	0.71	0.000				
35	0.602	0.498	0.033	0.047	0.019	0.005	0.00000	5.3	21.5	-0.680
	82.72	5.48	7.81	3.16	0.83	0.000				
<i>Sterilized milk.†</i>										
Fresh	0.608	0.528	0.018	0.029	0.010	0.023	0.0029	4.5	25.8	-0.735
	86.84	2.96	4.77	1.64	3.78	0.477				
35	0.637	0.547	0.012	0.027	0.027	0.024	0.00298	4.6	26.0	-0.615
	86.87	1.88	4.24	4.24	3.77	0.468				

†No analyses made during first four weeks of holding.

organisms, which had been killed by the formaldehyde. The subsequent decrease in acidity may be ascribed to either a neutralization or further decomposition of the lactic acid. In the *sterilized* milk the acidity remained constant during the entire period of holding.

Ravenel, Hastings and Hammer²⁷ report a decrease in the acidity of a clean milk and of a fair grade of commercial milk, held at -9°C. for a period of 203 days; and an increase, decidedly progressive, in the acidity of both the clean milk and the commercial milk held at 0°C. for that period of time.

²⁷ *Loc. cit.*

Lactose.

The lactose content of the *raw, untreated* milk decreased progressively, the greatest loss occurring during the earlier portion of the period of storage. In the *reinfectd, sterile* milk the decrease in lactose tended to parallel that of the raw untreated milk. The tendency was for the lactose to decrease but little, if at all, in the *formolized, raw* milk; a similar tendency was noted by Tice and Sherman²⁸ in raw formolized milk kept at the temperature of the laboratory. The *sterilized* milk showed no change in lactose content during the period of holding. The fermentation of the lactose with the formation of lactic acid was then largely, if not exclusively, due to the activity of bacteria.

Freezing point.

The decomposition of the constituents of the milk, especially the carbohydrate and the protein, with the formation of several small molecules from one large molecule must give rise to a higher molecular concentration of the solutes of the milk and, therefore, should be accompanied by a depression, or lowering, of the freezing point of the milk. The analytic findings are in perfect harmony with this theory. In the *raw, untreated* milk, the proteolysis and the fermentation of the lactose were accompanied by a lowering of the freezing point, which tended to be a progressive change. The changes in distribution of the nitrogen in the *formolized, raw* milk and the changes in protein and lactose in the *reinfectd, sterile* milk, were likewise accompanied by lowerings of the freezing point.

THE CHEMICAL CHANGES IN THE CREAM DURING HOLDING.

The fat constants.

The iodine number remained practically unchanged in all the experiments. The index of the refraction underwent no change in any of the samples. The Reichert-Meissl number showed no marked change.

In the *raw, untreated* cream, the saponification number, Hehner number and acid value seemingly increased progressively; the greatest rise in the saponification number occurred during the

²⁸ *Loc. cit.*

first week. In the *reinfected, sterile* cream, the saponification number showed an increase, followed by progressive decreases. The Hehner number and the acid value increased, with a tendency to do so progressively, the greatest increase being during the first week. In the *formolized, raw* cream the saponification number increased, especially during the first week, the Hehner number also increased with a tendency to do so progressively, although the greatest increment was during the first week. The acid value increased to a very slight extent. In the *sterilized* cream, the saponification number and the Hehner number increased, while the acid value suffered a slight decrease.

If the values of the saponification and Hehner numbers, and of the acid value obtained on the initial analysis, be compared with the highest values obtained in the subsequent analyses of each series, it will be observed that the greatest increase in acid value occurred in the reinfected sterile cream, the least in the formolized raw cream, with the raw untreated cream occupying an intermediate position. This would tend to show that the fat-splitting is of bacterial origin rather than due to enzymes of the cream.

The simultaneous increase in both Hehner and saponification numbers occurred even in the sterilized cream, showing that this reaction may depend simply on oxidation and the fine state of division of the butter-fat, possibly aided by a thermostable inorganic catalyst. Since this change was most pronounced in the formolized raw cream, native enzymes of the cream must also play a prominent rôle in its production.

The progressive decreases in the saponification number, accompanied by an increase in the Hehner number, in the reinfected sterile cream denote a type of fat decomposition, different from that observed in the sterilized, formolized raw, and raw untreated cream. Apparently, the raw untreated cream represents the resultant of the bacterial changes, revealed by the reinfected sterile cream, of oxidation and catalytic changes seen in the sterilized cream, and of the enzymic changes occurring in the formolized raw cream. The fine state of division of the butter-fat in the cream also, doubtless, plays a rôle in all the samples by exposing a large surface for oxidation.

TABLE II.

Chemical changes in raw and treated clean cream kept at 0°C.

PERIOD OF HOLD- ING AT 0°C.	FAT CONSTANTS								LECITHIN	FREEZING POINT
	Iodine num- ber	Saponification number	Acid value	Ester value	Per cent of free acid as oleic	Hehner num- ber	Reichert Meissl num- ber	Index of re- fraction at 35° C.		
<i>Raw untreated cream.</i>										
days								per cent		deg. C.
Fresh	32.0	206.0	0.5	205.5	0.25	76.8	31.1	1.4545	0.1159	-0.543
7	32.5	228.7	1.8	226.9	0.91	81.75	34.6	1.4546	0.1061	-0.620
14	33.1	221.2	1.2	220.0	0.60	85.0	31.0	1.4543	0.0917	-0.685
21	33.3	229.8	1.3	228.5	0.65	85.6	33.9	1.4543	0.1220	-0.720
28	31.4	226.5	2.1	224.4	1.06	83.0	33.0	1.4548	0.1388	-0.712
<i>Reinfected sterile cream.</i>										
Fresh	32.1	213.2	0.7	212.5	0.35	79.82	26.4	1.4546	0.1067	-0.565
7	33.1	228.6	2.6	226.0	1.31	83.6	34.7	1.4544	0.0967	-0.570
14	32.9	220.7	2.5	218.2	1.26	81.7	34.0	1.4544	0.1104	-0.600
21	33.4	183.9	2.8	181.1	1.41	85.4	30.0	1.4543	0.1106	-0.615
<i>Formolized raw cream.</i>										
Fresh	32.0	206.0	0.5	205.5	0.25	76.8	31.1	1.4545	0.1159	-0.543
7	32.8	233.7	0.52	233.2	0.26	83.6	27.4	1.4542	0.1249	-0.665
14	33.0	232.9	0.43	232.5	0.22	85.4	22.0	1.4542	0.1342	-0.655
21	32.5	238.6	0.33	238.3	0.17	85.9	35.5	1.4544	0.1529	-0.650
28	30.9	229.3	0.53	228.8	0.27	84.0	35.4	1.4535	0.1222	-0.660
<i>Sterilized cream.*</i>										
Fresh	32.1	213.2	0.7	212.5	0.35	79.82	26.4	1.4546	0.1067	-0.565
28	33.0	235.2	0.3	234.9	0.15	85.6		1.4551	0.1158	-0.622

*No analyses made during first three weeks of holding.

The lecithin.

This lipin apparently has not been decomposed in any of the four series of experiments.

Freezing point.

The *raw, untreated* cream showed a progressive lowering of the freezing point, as did the *reinfected, sterile* cream, though to a less degree. The *formolized, raw* cream showed a depression during the first week, then remained fairly constant. The *sterilized* cream showed a slight depression, but less than took place in the other samples. These changes were probably due to digestion of the protein and lactose of the cream, rather than to fat decomposition, for the Reichert-Meissl number, which may be accepted as a fair measure of soluble fatty acids, had undergone no marked increase; therefore, no soluble decomposition products of the butterfat had been formed to exert an influence on the freezing point.

ENZYMES OF THE MILK AND CREAM.

The results of the zymochemical experiments have been collected in Table III. In the *raw, untreated* milk and cream, reductases which attack methylene blue were apparently absent from both the fresh milk and the fresh cream, but were invariably present after the first week of holding at 0°C. Reductases which act upon methylene blue, plus formaldehyde, were present in both the fresh milk and the fresh cream and retained their activity throughout the period of storage. Oxidases which give rise to the oxidation of trikresol were always present in both the milk and the cream, while oxidases which are reactive toward guaiac were found in but three of the six milk analyses and in but two of the five cream analyses. In the *reinfected, sterile* milk and cream, reductases which destroy the color of methylene blue, as well as reductases which decolorize methylene blue, plus formaldehyde, were present in all the samples of milk save that tested after holding for one week, and were invariably present in the cream. Oxidases which produce oxidation of trikresol were always present in both the milk and the cream, while oxidases which cause a coloration of guaiac were always absent from the cream and were found only in the fresh

milk. In the *formolized*, raw milk and cream, reductases which decolorize methylene blue were absent from the fresh milk and from the final sample (held for thirty-five days at 0°C.) but were present in the other milk samples and invariably present in the cream. Reductases, which act on methylene blue plus formaldehyde, were always present in the cream and occurred in the fresh milk but were not found in any of the samples of milk which had been held at 0°C. for one or more weeks. Oxidases which attack trikresol were invariably present in both the milk and the cream, while oxidases which are reactive toward guaiac were invariably absent from both the milk and the cream.

Unfortunately, the data on catalase are incomplete. However, this enzyme apparently occurs as a true milk enzyme and is also secreted by the microorganisms. The raw untreated milk was always able to liberate more oxygen from hydrogen peroxide than was either the reinfected sterile or the formolized raw milk, yet this rule did not hold good in the case of the cream. At the end of two weeks' storage at 0°C. the cream showed greater catalytic activity than did the milk, but the reverse was true at the end of the third week.

To sum up, reductases which attack methylene blue were normal constituents of the raw untreated, reinfected sterile, and formolized raw samples of milk and of cream. Reductases which decolorize methylene blue in the presence of formaldehyde were invariably present in all three kinds of cream, were normally present in the raw untreated milk and in the reinfected sterile milk, and were usually absent from the formolized raw milk. It is, therefore, probable that the aldehyde reductases of the milk were of bacterial origin.

Oxidases, which give rise to an oxidation of trikresol, were invariably present in both milk and cream—raw untreated, reinfected sterile, and formolized raw. Oxidases, which produce a color with guaiac, were present in about half the experiments on raw untreated milk and cream, and were absent from all the other samples with the single exception of the fresh reinfected sterile milk.

These results point to the conclusion that the simple reductases and the trikresol oxidases of the milk may be enzymes native to the milk and may also be of bacterial origin. The aldehyde reductase

TABLE III.

Enzymes of raw and treated milk and cream kept at 0°C.

(The presence or absence of enzymes is expressed by the signs + and - respectively, except in the case of catalase where the figures represent cc. of oxygen evolved.)

SAMPLE NUMBER AND DESCRIPTION	PERIOD IN DAYS OF HOLDING SAMPLE AT 0°C	RAW UNTREATED					FORMULIZED RAW					REINJECTED STERILE				
		CATALASE	Methylene blue	Methylene blue plus formal- hyde	OXIDASES Guaiac	Triketosol	CATALASE	Methylene blue	Methylene blue plus formal- hyde	OXIDASES Guaiac	Triketosol	CATALASE	Methylene blue	Methylene blue plus formal- hyde	OXIDASES Guaiac	Triketosol
167 Milk.....	fresh		-	+	+	+		+	+	+	+		+	+	+	+
	7	2.65	+	+	+	+	1.60	+	+	+	+	0.55	+	+	+	+
	14	9.85	+	+	+	+	7.70	+	+	+	+	9.00	+	+	+	+
	21		+	+	+	+		+	+	+	+		+	+	+	+
	28	13.40	+	+	+	+		+	+	+	+		+	+	+	+
	35		+	+	+	+		+	+	+	+		+	+	+	+
168 Cream.....	fresh		-	+	+	+		+	+	+	+		+	+	+	+
	7	2.70	+	+	+	+	2.80	+	+	+	+	6.65	+	+	+	+
	14	3.80	+	+	+	+	5.70	+	+	+	+	5.70	+	+	+	+
	21		+	+	+	+		+	+	+	+		+	+	+	+
	28		+	+	+	+		+	+	+	+		+	+	+	+

of the milk was probably of bacterial origin, and apparently the guaiac oxidases arose from the same source. In the cream both varieties of reductase and the trikresol oxidases were apparently native enzymes and were also secreted by the microorganisms, while the guaiac oxidases were probably of bacterial origin. The catalase of both the milk and the cream was a true milk enzyme and was also due to the activity of microorganisms. All five enzymes—the two varieties of oxidase, the two varieties of reductase, and the catalase—retained their power to act as catalytic agents in spite of the prolonged exposure to a temperature of 0°C.

THE BACTERIAL CHANGES IN THE MILK DURING HOLDING.

Conn and Esten²⁹ report three experiments in which milk was kept at 1°C. for periods as long as 42 days, bacteriological analyses being made at intervals of 2 days to 9 days. During the first 6 or 8 days of holding, scarcely any bacterial development occurred, then the organisms increased steadily until very great numbers were present. Since the usual lactic acid organisms were not in the majority, the milk did not curd. A comparatively large number of liquefying organisms, and of neutral organisms, which produce neither acid nor alkali, were found in the milk. There was also a tendency for certain organisms to disappear during the period of holding.

Ravenel, Hastings and Hammer,³⁰ studied bacterial growth in milk held at low temperatures. The milk was of two kinds. "barn milk," the best milk obtainable, and "dairy milk," a fair commercial article. One sample of each kind of milk was kept at -9°C., and at 0°C., for a maximum period of 203 days, analyses being made at varying intervals. Up to and including 160 days, plates of lactose agar were sown and incubated at 37°C.; plates of plain gelatin were sown and incubated at 12 to 15°C. up to and including 203 days. During holding at -9°C., the number of organisms growing on agar at 37°C. remained fairly constant in both kinds of milk, the variations being insufficient to permit of any definite conclusions. The number of organisms developing

²⁹ Conn and Esten: *Sixteenth Annual Report of the Storrs Agricultural Experiment Station*, 1904, p. 27.

³⁰ *Loc. cit.*

on gelatin at 12 to 15°C. was fairly constant in the barn milk, but decreased considerably and with a marked progressive tendency in the dairy milk.

During holding at 0°C., the organisms growing on agar at 37°C. underwent a quiescent stage in both kinds of milk for six days, then increased progressively, in the barn milk to the end of the period of holding, in the dairy milk up to and including seventy-four days, afterward decreasing progressively. The organisms developing on gelatin at 12° to 15°C. were characterized by progressive increases to a maximum count followed by decreases; the maximum was attained in the dairy milk much earlier than in the barn milk.

In the *raw, untreated* milk on the plates incubated at 37°, the total count per cc. increased progressively up to the twenty-first day, then dropped rapidly and progressively. The anaerobes and facultatives rose during the first week, then fell; the maximum count, however, was at 21 days, after which a rapid progressive decrease occurred. The acid formers underwent a progressive increase up to the twenty-first day, then a rapid progressive decrease took place. On the plates incubated at 20°, the total count per cc. showed a progressive increase throughout the period of holding. There was a decided tendency for the anaerobes and facultatives to increase, the highest counts being at 21 and 28 days. The acid formers and the liquefiers increased progressively. On the plates incubated at 0°, the total count per cc. showed progressive increases and reached a maximum at 35 days. The anaerobes and facultatives tended to increase, and while this increase was not absolutely progressive, the highest counts were at 21 and 35 days. The acid formers were characterized by an increase, which, on the whole, was progressive.

In the *reinfected, sterile* milk, on the plates incubated at 37°, the total count per cc. rose to a maximum at 7 days, then suffered a progressive decrease throughout the experiment. The changes in the anaerobes and facultatives paralleled those of the total count, the maximum being at 7 days, after which there was a progressive decrease until no growth was obtained on the final analysis. The changes in the acid formers ran parallel to those of the total count and of the anaerobes and facultatives, the maximum at 7 days, then a progressive decrease to the end of the period of holding. On the plates incubated at 20°, the total count per cc. tended to rise

progressively, the maximum being at 28 days. The number of anaerobes and facultatives showed wide fluctuations, the greatest number being found at 14 and 35 days. The acid formers showed a decided trend to increase progressively, the maximum being at 28 days. The liquefiers increased progressively throughout the experiment. On the plates incubated at 0°, the total count per cc. rose progressively throughout the experiment. The anaerobes and facultatives and the acid formers showed a general tendency to increase progressively, reaching their highest counts at 28 days.

While a few organisms, including anaerobes and facultatives and acid formers, were present in the fresh *formolized raw* milk, yet in the subsequent analyses the plates were almost invariably sterile. The formolized raw milk of Tice and Sherman,³¹ which was kept at the temperature of the laboratory, at times contained liquefying cocci, which formed yellow colonies, as well as cocci and bacilli, which formed white colonies.

The *sterile* milk retained its sterility throughout the period of holding, as was demonstrated by platings made on the initial and final analyses.

Comparison of the raw and reinfected milk.

As a general rule, the total counts on the raw untreated milk were higher than on the reinfected sterile milk at all temperatures of incubation through the experiment, although the initial total count was practically the same. The initial counts of anaerobes and facultatives were fairly close in the two kinds of milk; during the first 2 weeks the reinfected sterile milk showed the highest count at all temperatures of incubation; at 3 weeks the raw untreated milk had the higher count at all temperatures of incubation; the raw untreated milk also continued to have the higher count at 37° throughout the rest of the experiment.

The initial count of acid formers differed in the two experiments, so the counts throughout the experiment can scarcely be compared. It may be noted that the relation of the acid formers to the total count varied throughout both series. At times the number of liquefiers was higher in the raw untreated milk, at times in the reinfected sterile milk.

³¹ *Loc. cit.*

Influence of temperature on the organisms.

On comparison of the counts, incubated at the three temperatures on the same analysis, the total count per cc. in the raw untreated milk had either 20° or 0° as the optimum temperature after the first week, in the reinfected sterile milk, 20° after the first week and 0° at the fifth week.

The anaerobes and facultatives in the raw untreated milk passed from an optimum temperature of 37° through 20° to 0°, while in the reinfected sterile milk the optimum growth was at either 20° or 0°.

The acid formers almost invariably had a maximum count on the plates grown at 20°.

Ratio of increase of the organisms.

The maximum count obtained during the period of holding with each group of organisms at each temperature of incubation is always compared with the count obtained with that group of organisms at the same temperature at the beginning of the experiment, using the latter figure as unity in the ratio.

RAW, UNTREATED MILK. *Total count per cc.* At 37° the maximum was attained at 21 days and was 463.9 times the count on the fresh milk. At 20° the maximum was at 35 days and the ratio of increase 1:3563.2; at 0° the maximum was at 35 days and the ratio of increase 1:290909.1. *Anaerobes and facultatives.* At 37° the maximum was at 21 days and the ratio of increase 1:118.9, at 20° the maximum was at 21 days and the ratio 1:340.0, at 0° the maximum was at 35 days and the ratio 1:533.3. *Acid formers.* At 37° the maximum was at 21 days and the ratio of increase 1:1913.0, at 20° the maximum was at 35 days and the ratio 1:12258.1, and at 0° the maximum was at 35 days and the ratio 1:357894.7. The maximum count of liquefiers was at 21 days and the ratio of increase 1:19000.0.

REINFECTED, STERILE MILK. *Total count per cc.* At 37° the maximum was at 7 days and the ratio of increase was 1:13.0, at 20° the maximum was at 28 days and the ratio 1:1437.5, at 0° the maximum was at 35 days and the ratio 1:19200.0. *Anaerobes and facultatives.* At 37° the maximum was at 7 days and the ratio of increase 1:34.6, at 20° the maximum was at 28 days and the ratio 1:505.9, at 0° the maximum was at 28 days and the ratio 1:454.5. *Acid formers.* At 37° the maximum was at 7 days and the ratio of increase 1:9.9, at 20° the maximum was at 28 days and the ratio 1:3035.7, and at 0° the maximum was at 28 days and the ratio 1:13333.3. The maximum count of liquefiers was at 35 days and the ratio of increase 1:5000.0.

Changes in Milk and Cream at 0°C.

TABLE IV. *Bacterial content and*
(The figures in Roman represent bacterial c

PERIOD OF KEEPING IN DAYS	PLATES INCUBATED AT °C	RAW UNTREATED MILK			
		Total count per cc.	Anaerobes and Facultatives per cc.	Acid Formers per cc.	Liquefiers per cc.
Fresh.....	37	97,000 1	18,500 1	11,500 1	
	20	87,000 1	10,000 1	15,500 1	1,000 1
	0	1,100 1	4,500 1	475 1	
	37	4,800,000 49.5	800,000 43.2	3,300,000 28.7	
7.....	20	9,500,000 109.2	360,000 36.0	4,700,000 303.2	1,500,000 1500.0
	0	10,000,000 9090.9	240,000 53.3	4,400,000 9263.2	
	37	13,000,000 134.0	85,000 4.6	7,600,000 660.9	
	20	87,000,000 1000.0	150,000 15.0	46,000,000 2967.7	2,700,000 2700.0
14.....	0	66,000,000 60,000.0	90,000 20.0	25,000,000 52,631.6	
	37	45,000,000 463.9	2,200,000 118.9	22,000,000 1913.0	
	20	180,000,000 2069.0	3,400,000 340.0	120,000,000 7741.9	19,000,000 19,000.0
	0	220,000,000 200,000.0	2,000,000 444.4	140,000,000 294,736.8	
21.....	37	170,000 1.8	20,000 1.1	80,000 7.0	
	20	180,000,000 2069.0	3,300,000 330.0	110,000,000 7096.8	
	0	150,000,000 136,363.6	1,200,000 266.6	67,000,000 141,052.6	
	37	14,000 0.14	4,000 0.22	8,000 0.70	
28.....	20	310,000,000 3563.2		190,000,000 12,258.1	15,000,000 15,000.0
	0	320,000,000 290.909.1	2,400,000 533.3	170,000,000 357,894.7	
	37				
	20				
35.....	0				
	37				
	20				
	0				

crease in milk kept at 0°C.

figures in *Italic* represent ratios of increase.)

REINFECTED STERILE MILK				FORMOLIZED RAW MILK
Total count per cc.	Anaerobes and FacultatIVES per cc.	Acid Formers per cc.	Liquefiers per cc.	Total count per cc.
100,000	26,000	91,000		400
<i>1</i>	<i>1</i>	<i>1</i>		
160,000	17,000	56,000	5,000	140
<i>1</i>	<i>1</i>	<i>1</i>	<i>1</i>	
12,500	4,400	9,000		0
<i>1</i>	<i>1</i>	<i>1</i>		
1,300,000	900,000	900,000		0
<i>13.0</i>	<i>34.6</i>	<i>9.9</i>		
1,500,000	600,000	1,000,000	20,000	0
<i>9.4</i>	<i>35.3</i>	<i>17.9</i>	<i>4.0</i>	
58,000		27,000		0
<i>4.6</i>		<i>3.0</i>		
900,000	120,000	330,000		0
<i>9.0</i>	<i>4.6</i>	<i>3.6</i>		
10,000,000	1,400,000	9,000,000	4,500,000	0
<i>62.5</i>	<i>82.4</i>	<i>160.7</i>	<i>900.0</i>	
9,100,000	460,000	4,600,000		0
<i>728.0</i>	<i>104.5</i>	<i>511.1</i>		
200,000	50,000	120,000		5
<i>2.0</i>	<i>1.9</i>	<i>1.3</i>		
72,000,000	330,000	54,000,000	12,000,000	
<i>450.0</i>	<i>19.4</i>	<i>964.3</i>	<i>2400.0</i>	
67,000,000	550,000	32,000,000		
<i>5400.0</i>	<i>125.0</i>	<i>3711.1</i>		
29,000	10,000	27,000		0
<i>0.29</i>	<i>0.38</i>	<i>0.30</i>		
230,000,000	8,600,000	170,000,000	14,000,000	0
<i>1437.5</i>	<i>505.9</i>	<i>3035.7</i>	<i>2800.0</i>	
180,000,000	2,000,000	120,000,000		0
<i>14,400.0</i>	<i>454.5</i>	<i>13,333.3</i>		
3,000	0	1,000		
<i>0.03</i>		<i>0.01</i>		
160,000,000	1,300,000	72,000,000	25,000,000	
<i>1000.0</i>	<i>76.5</i>	<i>1285.7</i>	<i>5000.0</i>	
240,000,000	1,100,000	62,000,000		
<i>19,200.0</i>	<i>250.0</i>	<i>6888.8</i>		

The general trend was for organisms which grow best at 37° to reach a maximum during the earlier stages of the period of holding, while those growing best at 20° and at 0° continued to increase and reached their highest values during the later stages of the experiment.

If the ratio of increase in the raw untreated milk at each period of analysis and at each temperature of incubation for each group of organisms be compared with the similar ratio in the reinfected sterile milk, it is seen that, in 42 of the 46 cases, 91.3 per cent, the ratio is higher in the raw milk than in the reinfected milk. Therefore, at 0°C. the raw milk has been the more suitable medium for the organisms and has given rise to a greater rate of proliferation.

The relative rate of growth of milk organisms in raw and pasteurized milk has been studied by several investigators. According to St. John and Pennington³² when raw clean milk and reinfected pasteurized clean milk of approximately the same bacterial content are held at the temperature of the room and of the ice box, the milk organisms proliferate more rapidly in the reinfected than in the raw sample. Rickards³³ likewise found that bacteria increase more rapidly in pasteurized than in unpasteurized milk at the temperature of the ice box. Ayers and Johnson³⁴ state that their results, obtained on milk kept at 10°C., "tend to prove that bacteria do increase approximately the same in pasteurized as in raw milk, provided their initial counts are practically alike."

The mode of preparing the reinfected sterile milk in the present research was the same procedure as used by St. John and Pennington in their study of reinfected pasteurized milk. The rate of bacterial growth was greater in the reinfected pasteurized milk than in the raw milk but was greater in the raw milk than in the reinfected sterile milk. On the other hand, in the experiments on cream described later in this paper, the rate of bacterial increase was greater in the reinfected sterile cream than in the raw cream. The following explanation is offered for these

³² *Loc. cit.*

³³ Rickards: *Amer. Journ. of Public Hygiene*, xix (New Series, v), p. 507, 1909.

³⁴ Ayers and Johnson: U. S. Dept. of Agric., Bureau of Animal Industry, Bulletin 126, 1910, p. 52.

phenomena. The pasteurized milk underwent no marked change in color and suffered no chemical change save, possibly, a partial coagulation of the lactalbumin; pasteurization had been attained by holding the milk at 79°C. for 20 minutes. The recent work of Rupp³⁵ demonstrates that the only appreciable chemical change produced in milk by pasteurization is a partial coagulation of the lactalbumin. Thus Rupp found that pasteurization of milk by holding at 71.1°C. for 30 minutes coagulated 30.87 per cent of the total lactalbumin of the milk. This investigator also noted that an increase in the temperature of pasteurization, the time factor remaining constant, gave rise to an increased coagulation of lactalbumin.

The sterilized milk had a marked light golden color; the major portion of the lactalbumin had been coagulated; and a portion of the lactose must have undergone decomposition, for the lactose content should have risen to a slight extent on account of the concentration during sterilization, yet the sterilized milk contained less lactose than did the fresh raw milk. One evidence of this decomposition of lactose is the greater acidity of the sterile than of the fresh raw milk on the initial analysis. The increase in lactic acid, however, is not sufficient to account for the decrease in lactose, and it appears probable that certain other decomposition products of the lactose may exert an inhibitory action on the bacterial growth in the reinfected sterile milk.

In the cream, the change in color as the result of sterilization was not noted, and moreover the lactose content of cream would be much less than that of milk, so the inhibitory substances would be formed to but a slight degree, if at all.

During the process of sterilization, however, the complex molecules of the organic constituents of the milk may possibly be rearranged in a manner which escapes detection by the ordinary methods of milk analysis, and the value of the milk as a nutrient medium for the rather fastidious organisms may be lessened, thus accounting for the slow rate of proliferation in the reinfected sterile milk.

³⁵ Rupp: U. S. Dept. of Agric., Bureau of Animal Industry, Bulletin 166, 1913.

The bacterial flora of the raw untreated milk and of the reinfected sterile milk.

The organisms which were isolated in pure culture from each sample of milk at each period of analysis are enumerated in Table V, according to the temperature of incubation of the plate on which each was found. The predominant species of organism at each temperature of incubation and each period of analysis of each sample is designated by a double asterisk (**), the species next numerous is designated by a single asterisk (*); at times, two organisms shared the premier position, in such cases both are designated by a double asterisk, and the single asterisk is omitted.

In the fresh *raw, untreated* milk, on the plates incubated at 37°, the predominant species was *Micrococcus ovalis* (Escherich); throughout the period of holding this organism tended to retain its position, although it twice shared that position with *Micrococcus aurantiacus* (Cohn), and once was displaced by the latter. On the plates incubated at 20°, *M. aurantiacus* held the premier position most of the time throughout the experiment, although it was once equalled and twice exceeded by *M. ovalis*. On the plates incubated at 0°, *M. aurantiacus* predominated until the very last analysis when it gave place to *M. ovalis*. At all three temperatures, whenever *M. ovalis* predominated, *M. aurantiacus* held the second place with respect to frequency of occurrence, and *vice versa*. Both these micrococci are acid formers. Apparently, *M. aurantiacus* preferred the lower temperatures of incubation, while *M. ovalis* became acclimated to those temperatures during the period of holding at 0°C. This process of acclimatization was also undergone by other organisms, thus *Bacterium flexuosum* was not found on the plates sown with fresh milk and incubated at 0°, but occurred on the 0° plates beginning with 7 days of holding and continuing to the end of the experiment.

On the other hand, some organisms soon disappeared from the milk during holding. Thus *Micrococcus acidi lactici* was recovered on the plates incubated at 37° from both the fresh milk and the milk after storage for 7 days, but thereafter could not be isolated from such plates. This organism was never found on the plates incubated at 20° and at 0°.

In the fresh *reinfected, sterile* milk, the predominant organism at 37° was *Micrococcus ovalis* (Escherich), at 20° and at 0° *Micro-*

TABLE V.

Organisms isolated in pure culture from raw untreated and reinfected sterile milk, kept at 0° C.

PERIOD IN DAYS OF HOLDING MILK AT 0° C.	FROM THE PLATES INCUBATED AT °C.	RAW UNTREATED MILK	REINFECTED STERILE MILK
Fresh	37	M. acidi lactici (Linder) M. albus liquifaciens *M. aurantiacus (Cohn) **M. ovalis (Escherich) S. flava	M. albus liquifaciens **M. ovalis (Escherich) S. farcinica S. flava *Penicillium
	20	M. albus liquifaciens **M. aurantiacus (Cohn) *M. ovalis (Escherich) B. flexuosum (Wright) S. flava	**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. flexuosum (Wright) S. flava Penicillium
	0	**M. aurantiacus (Cohn) *M. ovalis (Escherich)	**M. aurantiacus (Cohn) *M. ovalis (Escherich)
7	37	M. acidi lactici (Linder) M. albus liquifaciens *M. aurantiacus (Cohn) **M. ovalis (Escherich) B. flexuosum (Wright) B. detrudens (Wright)	M. albus liquifaciens *M. aurantiacus (Cohn) **M. ovalis (Escherich) B. flexuosum (Wright)
	20	**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. flexuosum (Wright) S. flava	M. albus liquifaciens **M. aurantiacus (Cohn) *M. ovalis (Escherich) B. flexuosum (Wright)
	0	**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. flexuosum (Wright) S. flava	*M. aurantiacus (Cohn) **M. ovalis (Escherich)
14	37	**M. aurantiacus (Cohn) **M. ovalis (Escherich) B. flexuosum (Wright)	M. aerius *M. aurantiacus (Cohn) **M. ovalis (Escherich)
	20	*M. aurantiacus (Cohn) **M. ovalis (Escherich) S. flava	*M. aurantiacus (Cohn) **M. ovalis (Escherich) B. flexuosum (Wright) S. flava

** The predominant species of organism at each temperature of incubation and at each period of analysis.

* The second most numerous species of organism at each temperature of incubation and at each period of analysis.

TABLE V.—Continued.

PERIOD IN DAYS OF HOLDING MILK AT 0°C.	FROM THE PLATES INCUBATED AT °C.	RAW UNTREATED MILK	REINFECTED STERILE MILK
14	0	** <i>M. aurantiacus</i> (Cohn) * <i>M. ovalis</i> (Escherich) <i>B. flexuosum</i> (Wright) <i>S. flava</i>	** <i>M. aurantiacus</i> (Cohn) * <i>M. ovalis</i> (Escherich) <i>B. flexuosum</i> (Wright) <i>S. flava</i>
	37	** <i>M. aurantiacus</i> (Cohn) * <i>M. ovalis</i> (Escherich) <i>B. flexuosum</i> (Wright) <i>S. flava</i>	** <i>M. aurantiacus</i> (Cohn) * <i>M. ovalis</i> (Escherich)
21	20	** <i>M. aurantiacus</i> (Cohn) ** <i>M. ovalis</i> (Escherich) <i>B. flexuosum</i> (Wright) <i>S. flava</i>	<i>M. albus liquifaciens</i> ** <i>M. aurantiacus</i> (Cohn) * <i>M. ovalis</i> (Escherich) <i>B. flexuosum</i> (Wright) <i>S. flava</i>
	0	** <i>M. aurantiacus</i> (Cohn) * <i>M. ovalis</i> (Escherich) <i>B. flexuosum</i> (Wright)	** <i>M. aurantiacus</i> (Cohn) * <i>M. ovalis</i> (Escherich) <i>B. flexuosum</i> (Wright)
28	37	** <i>M. aurantiacus</i> (Cohn) ** <i>M. ovalis</i> (Escherich)	** <i>M. aurantiacus</i> (Cohn) * <i>M. ovalis</i> (Escherich)
	20	* <i>M. aurantiacus</i> (Cohn) ** <i>M. ovalis</i> (Escherich) <i>S. flava</i>	<i>M. aurantiacus</i> (Cohn) ** <i>M. ovalis</i> (Escherich) * <i>B. flexuosum</i> (Wright)
	0	** <i>M. aurantiacus</i> (Cohn) * <i>M. ovalis</i> (Escherich) <i>B. flexuosum</i> (Wright)	* <i>M. aurantiacus</i> (Cohn) ** <i>M. ovalis</i> (Escherich) <i>B. flexuosum</i> (Wright)
35	37	* <i>M. aurantiacus</i> (Cohn) ** <i>M. ovalis</i> (Escherich) <i>S. flava</i>	** <i>M. aurantiacus</i> (Cohn) * <i>M. ovalis</i> (Escherich) * <i>S. flava</i>
	20	** <i>M. aurantiacus</i> (Cohn) * <i>M. ovalis</i> (Escherich) <i>B. flexuosum</i> (Wright) <i>S. flava</i>	** <i>M. aurantiacus</i> (Cohn) * <i>M. ovalis</i> (Escherich) <i>B. flexuosum</i> (Wright) <i>S. flava</i>
	0	* <i>M. aurantiacus</i> (Cohn) ** <i>M. ovalis</i> (Escherich) <i>B. flexuosum</i> (Wright)	** <i>M. aurantiacus</i> (Cohn) * <i>M. ovalis</i> (Escherich)

**The predominant species of organism at each temperature of incubation and at each period of analysis.

*The second most numerous species of organism at each temperature of incubation and at each period of analysis.

coccus aurantiacus (Cohn). Throughout the period of holding, at each of the three temperatures of incubation, *M. ovalis* predominated twice, *M. aurantiacus* thrice. The two lower temperatures, at the beginning of the experiment offered a more favorable environment to *M. aurantiacus* than to *M. ovalis*, yet the latter exhibited a tendency to become acclimated during holding of the milk at 0°, as was shown by its predominance at 28 days on the plates incubated at 20° and at 0°. These two acid-forming micrococci—*M. aurantiacus* and *M. ovalis*—almost invariably were the two more numerous species. On the plates at 37°, however, the second place in the fresh sample was occupied by *Penicillium*, and in the final sample was shared by *M. ovalis* and *Streptothrix flava*, while on the plates at 20° the second place at 28 days was occupied by *Bacterium flexuosum*. The tendency to become acclimated to the low temperature was also shown by *Bacterium flexuosum* which disappeared from the 37° plates after the first week of holding, appeared on the 20° plates beginning with the first week and on the 0° plates beginning with the second week. The opposite tendency—to disappear entirely from the milk during holding—was exhibited by *Streptothrix farcinica* which was found only on the 37° plates of the fresh sample; it failed to develop on the 20° and 0° plates of that sample and was never found during the subsequent analyses..

THE BACTERIAL CHANGES IN THE CREAM DURING HOLDING.

In the raw, untreated cream, on the plates incubated at 37° the total count per cc. increased progressively up to 14 days, then decreased; the anaerobes and facultatives rose during the first week, then decreased; the acid formers underwent a progressive increase for 14 days, then decreased. On the plates incubated at 20°, the total count per cc. increased irregularly to a maximum at 21 days, followed by a decrease on the final analysis; the anaerobes and facultatives attained their highest value at 7 days, then exhibited a marked tendency to decrease progressively; the acid formers reached their highest values at 7 and 21 days, there being a trend toward a maximum during the middle of the experiment, then a decrease toward the close of the experiment; the liquefiers increased more or less regularly throughout the entire experiment. On the

TABLE VI. *Bacterial con*
(The figures in Roman represent bac

PERIOD OF KEEPING IN DAYS	PLATES INCUBATED AT °C	RAW UNTREATED CREAM			
		Total count per cc.	Anaerobes and FacultatIVES per cc.	Acid Formers per cc.	Liquefiers per cc.
Fresh.....	37	5,900,000 1	1,400,000 1	550,000 1	
	20	6,100,000 1	1,700,000 1	1,800,000 1	320,000 1
	0	2,200,000 1	56,000 1	1,100,000 1	
7.....	37	15,000,000 2.5	5,000,000 3.6	10,000,000 18.2	
	20	97,000,000 15.9	29,000,000 17.1	72,000,000 40.0	7,000,000 21.9
	0	23,000,000 10.5	4,000,000 71.4	5,000,000 4.5	
14.....	37	58,000,000 9.8	3,300,000 2.4	20,000,000 36.4	
	20	53,000,000 8.7	8,000,000 4.7	47,000,000 26.1	3,000,000 9.4
	0	12,000,000 5.5	1,200,000 21.4	5,000,000 4.5	
21.....	37	320,000 0.05		50,000 0.09	
	20	120,000,000 19.7	290,000 0.17	70,000,000 38.9	14,000,000 43.8
	0	120,000,000 54.5	12,000,000 214.3	26,000,000 23.6	
28.....	37	420,000 0.07	130,000 0.09	180,000 0.33	
	20	10,000,000 1.6	1,000,000 0.59	3,600,000 2.0	19,000,000 59.4
	0				

plates incubated at 0°, the *total count per cc.*, *anaerobes and facultatives*, and *acid formers* all showed a distinct tendency to increase progressively during the entire period of holding.

In the *reinfectd, sterile cream*, on the plates incubated at 37°, the *total count per cc.*, *anaerobes and facultatives* and *acid formers* rose progressively up to 14 days, then decreased on the final analy-

in cream kept at 0°C.

(in Italic represent ratios of increase.)

REINFECTED STERILE CREAM				FORMOLIZED RAW CREAM
Total count per cc.	Anaerobes and Facultatives per cc.	Acid Formers per cc.	Liquefiers per cc.	Total count per cc.
850,000	15,000	38,000		160,000
<i>1</i>	<i>1</i>	<i>1</i>		
1,300,000	650,000	750,000	33,500	0
<i>1</i>	<i>1</i>	<i>1</i>	<i>1</i>	
13,000	2,600	5,600		0
<i>1</i>	<i>1</i>	<i>1</i>		
11,000,000	600,000	8,600,000		0
<i>12.9</i>	<i>40.0</i>	<i>226.3</i>		
48,000,000	9,000,000	26,000,000	15,000,000	0
<i>36.9</i>	<i>13.8</i>	<i>34.7</i>	<i>447.8</i>	
20,000,000		8,400,000		0
<i>1538.5</i>		<i>1500.0</i>		
19,000,000	4,000,000	9,000,000		0
<i>22.4</i>	<i>266.7</i>	<i>236.8</i>		
38,000,000	16,000,000	35,000,000	2,000,000	
<i>29.2</i>	<i>24.6</i>	<i>46.7</i>	<i>59.7</i>	
7,800,000	1,000,000	3,700,000		
<i>600.0</i>	<i>384.6</i>	<i>660.7</i>		
40,000		20,000		3
<i>0.05</i>		<i>0.53</i>		
130,000,000	160,000	56,000,000	28,000,000	
<i>100.0</i>	<i>0.25</i>	<i>74.7</i>	<i>835.8</i>	
120,000,000	1,500,000	30,000,000		
<i>9230.8</i>	<i>576.9</i>	<i>5357.1</i>		
				1

sis. On the plates incubated at 20°, the *total count per cc.*, *acid formers* and *liquefiers* rose more or less progressively to their highest values on the concluding day of the experiment; the *anaerobes and facultatives* rose progressively during the first fortnight of holding, then suffered an enormous decrease during the third and last week in storage. On the plates incubated at 0°, the *total count per cc.*,

anaerobes and facultatives and *acid formers* rose with more or less regularity to maximum values on the twenty-first and concluding day of the experiment.

In the fresh *formolized*, *raw* cream, organisms were present, but subsequent analyses demonstrated that the cream had become sterile.

The *sterile* cream retained its sterility throughout the period of holding, as was demonstrated by platings made on the initial and final analyses.

Since the initial counts for all groups of organisms differ widely in the raw untreated and reinfected sterile cream, comparison of the counts themselves at different periods of the experiment cannot be made. A comparison of the ratio of increase of the various groups of organisms in the two kinds of cream, however, will be made during the discussion of those ratios (see page 363).

As a rule, the optimum temperature of incubation for all groups of bacteria remained at 20° throughout the entire period of study.

Ratio of increase of the organisms.

The maximum count obtained during the period of holding with each group of organisms at each temperature of incubation is always compared with the count obtained with that group of organisms at the same temperature of incubation at the beginning of the experiment, using the latter figure as unity in the ratio.

RAW, UNTREATED CREAM. *Total count per cc.* At 37° the maximum ratio of increase was attained at 14 days and was 9.8 times the count on the fresh cream. At 21 days the maximum ratio at 20° (1:19.7) and at 0° (1:54.5) was reached. *Anaerobes and facultatives.* The maximum increase at 37° (1:3.6) and at 20° (1:17.1) occurred at 7 days, while the maximum for 0° (1:214.3) was attained at 21 days. *Acid formers.* At 37° the maximum was reached at 14 days with the ratio 1:36.4, while at 0° the maximum was at 21 days with the ratio 1:21.7; the maximum ratio at 20° was 1:40.0 at 7 days, and was closely followed by the ratio 1:38.9 at 21 days. *Liquefiers* reached their highest value at 28 days with a ratio of increase 1:59.4.

REINFECTED, STERILE CREAM. *Total count per cc.* The maximum increase at 37° (1:22.4) occurred at 14 days, the maximum at 20° (1:100.0) and at 0° (1:9230.8) took place at 21 days. *Anaerobes and facultatives.* The maximum ratio of increase at 37° (1:266.7) and at 20° (1:24.6) was attained at 14 days, while the maximum at 0° (1:567.9) was at 21 days. *Acid formers.* At 37° the greatest increase (1:236.8) was attained at 14 days, while the maximum increase at 20° (1:74.7) and at 0° (1:5371.1) was found at 21 days. The greatest ratio of increase for *liquefiers* was 1:835.8 at 21 days.

The organisms which proliferate best at 37° always reached their highest numbers by the fourteenth day of holding, while the maximum ratio of increase of the organisms which grow best at 20° and at 0° almost invariably occurred on the twenty-first day.

Upon comparison of the ratio of increase in the raw untreated cream at each period of analysis and at each temperature of incubation for each group of organisms with the similar ratio in the reinfected sterile cream, it is seen that, in 25 of the 27 cases, 92.6 per cent, the ratio is higher in the reinfected cream than in the raw cream. Therefore, at 0°C., the reinfected cream has been a more suitable medium for bacterial reproduction and has given rise to a greater rate of growth.

The bacterial flora of the raw untreated cream and of the reinfected sterile cream.

The mode of tabulation of the organisms is the same as was used for the bacterial flora of the milk. In the fresh raw, untreated cream, on the plates incubated at 37° and at 20°, *Micrococcus aurantiacus* (Cohn) predominated, while on the plates incubated at 0° *Micrococcus ovalis* (Escherich) occupied that position. During the period of holding, on the plates at 37°, *M. ovalis* predominated until the last analysis when it gave place to *M. aurantiacus*; on the plates at 20°, *M. ovalis* occupied the first position but was displaced by *M. aurantiacus* during the last two weeks of the experiment; on the plates at 0°, *M. aurantiacus* was the predominant organism throughout the entire period of holding. The first and second positions were almost invariably held by the two acid-forming micrococci, *M. aurantiacus* and *M. ovalis*; the only exception occurred at 14 days on the plates incubated at 20° where *Bacterium aerophilum* occupied second place. On the whole the storage at a temperature of 0°C. exerted a favorable influence on *M. aurantiacus* and an unfavorable influence on *M. ovalis*, for the former gradually displaced the latter even on the plates grown at 37°. The disappearance of certain organisms during holding was illustrated by *M. acidi lactis* and *Bacillus detrudens*, which were isolated from plates sown with fresh cream and incubated at 37°; these organisms, however, were never found in the cream during the period of holding.

TABLE VII.

Organisms isolated in pure culture from raw untreated and reinfected sterile cream, kept at 0°C.

PERIOD IN DAYS OF HOLDING CREAM AT 0°C.	FROM THE PLATES INCUBATED AT °C.	RAW UNTREATED CREAM	REINFECTED STERILE CREAM
Fresh	37	M. acidi lactis **M. aurantiacus (Cohn) *M. ovalis (Escherich) B. flexuosum (Wright) B. detrudens (Wright) S. Rosenbachii (Kruse)	**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. detrudens (Wright) S. Rosenbachii (Kruse)
	20	M. acidi lactici **M. aurantiacus (Cohn) *M. ovalis (Escherich) B. aerophilum B. flexuosum (Wright) S. flava	*M. aurantiacus (Cohn) **M. ovalis (Escherich) B. aerophilum B. flexuosum (Wright) S. farcinica S. Rosenbachii (Kruse)
	0	*M. aurantiacus (Cohn) **M. ovalis (Escherich) B. aerophilum B. flexuosum (Wright)	**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. aerophilum B. flexuosum (Wright)
7	37	M. alvi *M. aurantiacus (Cohn) **M. ovalis (Escherich) S. Rosenbachii (Kruse)	M. acidi lactici M. alvi *M. aurantiacus (Cohn) **M. ovalis (Escherich) S. Rosenbachii (Kruse)
	20	*M. aurantiacus (Cohn) **M. ovalis (Escherich) B. flexuosum (Wright) S. flava	M. aurantiacus (Cohn) **M. ovalis (Escherich) *S. flava
	0	**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. aerophilum S. flava	**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. aerophilum

* The predominant species of organism at each temperature of incubation and at each period of analysis.

* The second most numerous species of organism at each temperature of incubation and at each period of analysis.

TABLE VII—Continued.

PERIOD IN DAYS OF HOLDING CREAM AT 0°C.	FROM THE PLATES INCUBATED AT °C.	RAW UNTREATED CREAM	REINFECTED STERILE CREAM
14	37	* <i>M. aurantiacus</i> (Cohn) ** <i>M. ovalis</i> (Escherich) <i>B. aerophilum</i> <i>B. flexuosum</i> (Wright)	** <i>M. aurantiacus</i> (Cohn) * <i>M. ovalis</i> (Escherich) <i>B. aerophilum</i> <i>B. flexuosum</i> (Wright) <i>S. flava</i>
	20	* <i>M. aurantiacus</i> (Cohn) ** <i>M. ovalis</i> (Escherich) <i>B. aerophilum</i> <i>B. flexuosum</i> (Wright) <i>S. flava</i>	* <i>M. aurantiacus</i> (Cohn) ** <i>M. ovalis</i> (Escherich) <i>B. aerophilum</i> <i>S. flava</i> <i>S. Rosenbachii</i> (Kruse)
	0	** <i>M. aurantiacus</i> (Cohn) * <i>M. ovalis</i> (Escherich) <i>B. aerophilum</i> <i>S. flava</i>	** <i>M. aurantiacus</i> (Cohn) * <i>M. ovalis</i> (Escherich) <i>B. aerophilum</i>
21	37	* <i>M. aurantiacus</i> (Cohn) ** <i>M. ovalis</i> (Escherich) <i>B. aerophilum</i>	<i>M. acidi lactici</i> ** <i>M. aurantiacus</i> (Cohn) <i>M. ovalis</i> (Escherich) * <i>B. aerophilum</i> <i>S. Rosenbachii</i> (Kruse)
	20	** <i>M. aurantiacus</i> (Cohn) * <i>M. ovalis</i> (Escherich) <i>B. aerophilum</i>	** <i>M. aurantiacus</i> (Cohn) * <i>M. ovalis</i> (Escherich) <i>B. aerophilum</i> <i>B. flexuosum</i> (Wright) <i>S. flava</i>
	0	** <i>M. aurantiacus</i> (Cohn) * <i>M. ovalis</i> (Escherich) <i>B. aerophilum</i> <i>B. flexuosum</i> (Wright)	** <i>M. aurantiacus</i> (Cohn) * <i>M. ovalis</i> (Escherich) <i>B. aerophilum</i> <i>B. flexuosum</i> (Wright)
28	37	** <i>M. aurantiacus</i> (Cohn) * <i>M. ovalis</i> (Escherich) <i>B. aerophilum</i>	
	20	** <i>M. aurantiacus</i> (Cohn) * <i>M. ovalis</i> (Escherich) <i>B. aerophilum</i>	

** The predominant species of organism at each temperature of incubation and at each period of analysis.

* The second most numerous species of organism at each temperature of incubation and at each period of analysis.

In the *reinfect*ed, *sterile* cream, on the plates at 37°, *Micrococcus aurantiacus* (Cohn) predominated in the fresh sample and held that position throughout the entire experiment save at 7 days, where it gave place to *Micrococcus ovalis* (Escherich). On the plates at 20°, *M. ovalis* was the predominating species save on the final analysis when it was displaced by *M. aurantiacus*. On the plates at 0°, *M. aurantiacus* held the first position throughout the entire period of holding. At all three temperatures of incubation, the first and second positions were usually shared by *M. ovalis* and *M. aurantiacus*; however, the second position at 21 days on the plates at 37° was held by *Bacterium aerophilum*, and at 7 days on the plates at 20° by *Streptothrix flava*. The trend for some organisms to disappear during holding was illustrated by *Bacillus destrudens*, which was isolated from the plates sown with the fresh sample and incubated at 37°; at subsequent periods of analysis, the organism was never found.

GENERAL CONCLUSIONS.

In milk and cream which are held at a temperature of 0°C., the following phenomena are noted:

The proteolysis of the casein is, primarily, of bacterial origin.

The proteolysis of the lactalbumin is due, primarily, to native enzymes of the milk.

The bacterial flora and the native milk enzymes by their combined action give rise to more rapid proteolytic changes than are produced by either agent alone.

The general trend of the proteolysis by bacteria, by enzymes, and by the combined action of these two agents, involves a breaking down of the true proteins and their passage through caseose and peptone to amino-acids.

The fermentation of the lactose with the formation of lactic acid is largely, if not exclusively, due to bacterial action.

The digestion of the protein, the fermentation of the lactose and the increase in acidity are progressive changes, and are accompanied by more or less progressive lowerings of the freezing point of the milk.

The depression of the freezing point of the cream is to be ascribed to chemical changes in its protein and lactose.

The lecithin of the cream was not decomposed during the period of holding.

The iodine number and the index of refraction of the butter-fat remained unchanged, while the Reichert-Meissl number underwent no marked increase or decrease in any of the samples.

The hydrolysis of the fat and the increase in acid value, which is usually progressive, are due to the action of bacteria.

The Hehner number always becomes greater; the saponification number usually increases, although it underwent progressive decreases in the reinfected sterile cream. The increase in Hehner number, accompanied by a decrease in saponification number, in the reinfected sterile cream is to be ascribed to bacterial action. The simultaneous increase in the two constants in the sterilized cream is doubtless due to oxidation and the fine state of division of the butter-fat in the cream, possibly aided by a thermostable inorganic catalyst; these same causes, plus the action of the native enzymes, must give rise to the simultaneous increase in the Hehner and saponification numbers of the formolized raw cream; while the simultaneous rise in these two constants in the raw untreated cream are the resultant of the action of bacteria, native enzymes, oxidation, inorganic catalysts and fine state of division of the butter-fat.

The simple reductases and the trikresol oxidases of the *milk* may be native enzymes and may also be of bacterial origin, while the aldehyde reductase and the guaiac oxidases, apparently, are of bacterial origin.

Both varieties of reductase and the trikresol oxidases are native enzymes of the *cream* and are also secreted by the microorganisms, while the guaiac oxidases probably have their origin in bacterial action.

The catalase of both the milk and the cream is a native enzyme and is also due to the activity of microorganisms.

The two varieties of oxidase, the two varieties of reductase and the catalase retain their activity in spite of the prolonged exposure to a temperature of 0°C.

During the holding at 0°C., the organisms of the raw untreated and reinfected sterile milk and cream undergo an increase, which is most striking in the raw untreated milk.

In both milk and cream, the organisms which proliferate best at 37°C. reach a maximum growth during the earlier stages of the period of holding, while those growing best at 20°C. and at 0°C. continue to increase and attain their highest values during the later stages of the experiment.

Some organisms disappear during holding, others become acclimated to the lower temperatures of incubation.

The total count per cc. at 37° was practically the same in the raw untreated and reinfected sterile milk; in over 90 per cent of the subsequent determinations the rate of increase of the various groups of organisms was greater in the raw than in the reinfected sample.

In over 90 per cent of the determinations made during the period of holding, the rate of increase of the various groups of organisms was greater in the reinfected sterile cream than in the raw untreated cream.

Almost invariably *Micrococcus aurantiacus* (Cohn), and *Micrococcus ovalis* (Escherich), which belong to the group of acid-formers, were the predominant organisms of both raw untreated and reinfected sterile milk and cream.

THE REACTION OF SOME PURINE, PYRIMIDINE, AND HYDANTOIN DERIVATIVES WITH THE URIC ACID AND PHENOL REAGENTS OF FOLIN AND DENIS.

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(Received for publication, October 10, 1913.)

In the course of an investigation by one of us¹ on the behavior of certain thioderivatives of hydantoin in the animal organism, it was observed that the substances studied reacted with the reagents of Folin and Denis² with the development of the blue color described as typical for uric acid. The possibility of using this reaction for the detection of the compounds under investigation at once suggested itself, and an examination of various related substances in regard to their behavior toward the new reagents was undertaken. Recently Funk and Macallum³ have studied the reaction with certain purines, pyrimidines, and related substances of biochemical importance. Inasmuch as our results supplement and extend the work of these investigators, we offer them in the hope that they may prove of value in pointing out a line of attack for the solution of the problem of the chemical basis of the reactions. For many of the compounds studied we are indebted to Profs. Lafayette B. Mendel and T. B. Johnson, who have placed them at our disposal.

With the exception of thiourea no substance was observed to react typically with the phenol reagent, which does not contain a phenol group or react with the uric acid reagent. *N*-*i*-methyl-tyrosine reacts positively with the phenol reagent, but the related

¹ Lewis: this *Journal*, xiv, pp. 245-56, 1913.

² Folin and Denis: this *Journal*, xii, p. 239, 1912.

³ Funk and Macallum: *Biochem. Journ.*, vii, pp. 356-58, 1913.

TABLE I.

	URIC ACID REAGENT	PHENOL REAGENT
1. Hydantoin.....	—	—
2. Hydantoin-4-acetic acid.....	—	—
3. Hydantoin-4-propionic acid.....	—	—
4. <i>p</i> -Hydroxybenzylhydantoin.....	—	+++
5. <i>p</i> -Aminobenzylhydantoin.....	—	—
6. Parabanic acid.....	—	—
7. Aminocarboxyhydantoin.....	+++	+++
8. 2-Thiohydantoin.....	+++	+++
9. 2-Thio-4-methylhydantoin.....	+++	+++
10. 2-Thiohydantoin-4-acetic acid.....	+++	+++
11. 2-Thio-4-benzalhydantoin.....	—	—
12. 2-Thio-4-benzylhydantoin.....	+++	+++
13. 1-Phenyl-2-thio-4-anisalhydantoin.....	—	—
14. 1-Phenyl-2-thio-4-anisylhydantoin.....	+++	+++
15. 1-Phenyl-2-thio-4-cinnamalhydantoin.....	—	—
16. 1-Phenyl-2-thio-4-furfuralhydantoin.....	—	—
17. 1-Phenyl-2-thiohydantoin.....	+++	+++
18. 3-Phenyl-2-thiohydantoin.....	+++	+++
19. 1,3-Diphenyl-2-thiohydantoin.....	+++	+++
20. 1-Phenyl-2-thio-4-piperonalhydantoin.....	—	—
<i>Purine derivatives.</i>		
21. 2,8-Dioxyurine.....	—	—
22. 2,8-Dioxy-6-methylpurine.....	—	—
23. 2,8-Dioxy-6,9-dimethylpurine.....	—	—
24. 2,8-Dioxy-9-methylpurine.....	—	—
25. 2,8-Dioxy-1,9-dimethylpurine.....	—	—
26. 2-Oxy-9-methylpurine.....	—	—
27. 2-Oxy-6,9-dimethylpurine.....	—	—
28. 2-Oxy-6,8,9-trimethylpurine.....	—	—
29. 2,6-Dioxy-8-methylpurine.....	—	—
30. 2,6-Dioxy-1,7-dimethylpurine.....	—	—
31. 2,6-Dioxy-1,3-dimethylpurine.....	—	—
32. 2,6-Dioxy-1,3,7-trimethylpurine.....	—	—
33. Sodium urooxanate.....	—	—
34. 2-Thio-6-oxypurine.....	+++	+++
35. 2,8-Dithio-6-oxypurine.....	+++	+++
36. 2-Thio-6,8-dioxypurine.....	+++	+++
37. 2-Thioglycollic acid-6-oxypurine.....	—	—
38. 2,8-Dithioglycollic acid-6-oxypurine.....	—	+
39. 2-Thioglycollic acid-6,8-dioxypurine.....	—	+

TABLE I.—Continued.
Pyrimidine derivatives.

	URIC ACID REAGENT	PHENOL REAGENT
40. Cytosine.....	—	
41. Isocytosine.....	—	
42. 6-Methyleytosine.....	—	
43. Thymine.....	—	
44. 1-Methylthymine.....	—	
45. 1,3-Dimethylthymine.....	—	
46. Diethylbarbituric acid (Veronal).....	—	
47. Barbituric acid.....	—	
48. Violuric acid.....	—	
49. 2-Thiovioluric acid.....	+++	+++
50. Cyanacetylguanidine.....	—	
51. 2-Phenylamino-6-oxypyrimidine.....	—	
52. 2-Ethylmercaptopyrimidine.....	—	
53. 2,4-Diamino-6-oxypyrimidine sulphate.....	—	
54. 2,4,5-Triamino-6-oxypyrimidine.....	+++	
55. Malonylguanidine.....	—	
56. 5-Aminomalonylguanidine hydrochloride.....	+++	
57. 2-Thiopseudouric acid.....	+++	+++
58. 2-Thiouramil.....	+++	+++

Miscellaneous compounds.

59. Thiotyrosine.....	+++	+++
60. Thiourea.....	—	+++
61. Thiobenzamide.....	+	+
62. N-methyldiiodtyrosine.....	—	+++
63. N- <i>i</i> -methyltyrosine.....	—	+++
64. α -Methylamino- β - <i>p</i> -methoxyphenylpropionic acid.....	—	—
65. Glycollic acid.....	—	—
66. Allantoin.....	—	—
67. Malonaminourethane.....	+++	+++
68. Dithiodimethylpiperazine.....	+++	++
69. Benzoylthiourea.....	—	—
70. Benzoylphenylthiourea.....	—	—

α -methylamin o β aramethoxyphenylpropionic acid in which a methyl group has been substituted for the hydrogen of the hydroxyl group fails to give the reaction. N-methyldiiodtyrosine, which does not respond to Millon's test, gives a positive reaction with the

phenol reagent, indicating that it is not a necessary condition for this reaction as for Millon's that the position ortho to the hydroxyl group in the benzene ring shall be unsubstituted. Thiotyrosine reacts positively with the phenol reagent, but inasmuch as it also reacts positively with the uric acid reagent and as will be discussed later sulphur seems to play a rôle here, no conclusion as to the effect of $-SH$ groups replacing $-OH$ groups can be drawn. Of the cyclic compounds examined which contained no sulphur, only three, 4-aminocarboxyhydantoin, 2,4,5-triamino-6-oxypyrimidine, and 5-aminomalonylguanidine react positively with the uric acid reagent. The striking feature possessed by these three compounds in common, in contrast with the closely related hydantoin, 2,4-diamino-6-oxypyrimidine, and malonylguanidine, none of which gives a positive reaction, is the presence of an amino group. In 2,4,5-triamino-6-oxypyrimidine and 5-malonylguanidine, the amino group which determines the reaction is in the 5-position as is evidenced by the inactivity of the 2,4-diamino compound and malonylguanidine respectively. It is interesting to note that malonaminourethane of which 4-aminocarboxyhydantoin is the cyclic anhydride is the only acyclic compound free from sulphur which reacted positively with the uric acid reagent. The activity of amino groups is in harmony with the observations of Folin and Denis and Funk and Macallum, that monohydric phenols do not react with the uric acid reagent unless an amino group is present in the benzene ring (*e. g.*, 2- or 3-amino-tyrosine).

With the exception of the thiopurines, none of the purines studied react positively. Neither xanthine nor its isomer, nor any of their methyl derivatives react. Allantoin and uroxic acid, both oxidation products of uric acid, fail to react.

The presence of sulphur replacing oxygen in the 2-position of a purine, pyrimidine, or hydantoin, gives rise to a positive reaction with both reagents (*cf.* 8-20, 34-36, 49, 57, 58). In the case of the condensation products of thiohydantoins with aldehydes, derivatives in which the carbon in the 4-position is unsaturated (11, 13, 15, 16, 20) the reaction is negative. When reduced the resulting products react positively (*cf.* 11 and 12, 13 and 14). That sulphur replacing oxygen rather than mercapto sulphur is a condition for a positive test is shown by the negative reactions

of the mercapto derivatives of the purines and pyrimidines (37-39, 52). Dithiodimethylpiperazine, the anhydride of the thiopoly-peptide, thioalanyl-thioalanine, reacts positively. Although thio-urea gives the test with the phenol reagent, neither of the two substituted thioureas (69, 70) gives a positive reaction.

The suggestion made by Funk and Macallum that in the purines the substitution of the hydrogen atoms of the ring lessens or destroys the power to react with the uric acid reagent, does not hold for the hydantoin ring. Those thiohydantoins in which substitution has occurred on the 1- or 3-carbon atom or on both (17-19), react as readily with the reagents as do the unsubstituted compounds.



THE FORMATION OF GLUCOSE FROM PROPIONIC ACID IN DIABETES MELLITUS.

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(Received for publication, October 15, 1913.)

It has been shown by Ringer¹ that the administration of propionic acid to phlorhizinized dogs is followed by the elimination of "extra glucose" equal in amount to that capable of being formed from the propionic acid if all three carbon atoms are used in the formation of glucose.

Shortly after the publication of this work we had under observation a patient who exhibited a G : N ratio of about 3.5. The case seemed to be eminently suitable for the comparison of glucose formation in phlorhizin glycosuria and in diabetes mellitus. Propionic acid being readily obtainable it seemed desirable to ascertain if it would give rise to glucose. This was found to be the case. The experiment was subsequently repeated upon the same patient and also upon two others with less severe forms of diabetes. In one patient, who had only a slight diabetes, the administration of propionic acid did not increase the excretion of glucose. Otherwise, a distinct rise was observed in every experiment. The excretion of acetone and β -hydroxybutyric acid was followed in some of the experiments. In only one of these was there an increase in the amount of these substances eliminated and in this case there was a further rise on the following day. Four typical experiments are summarized in the accompanying tables.

The propionic acid was prepared by Kahlbaum. The analytical methods employed were the Kjeldahl for nitrogen, the Benedict for glucose and the Shaffer for acetone and β -hydroxybutyric acid. The figures for the carbohydrate in the diet were calculated from the data given in Bulletin 28, U. S. Department of Agriculture.

¹ Ringer: *This Journal*, xii, p. 511, 1912.

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As is evident from the tables, in the first experiment the "extra glucose" was almost exactly equivalent in amount to the propionic acid ingested. Later when the G:N ratio was lower, indicating an increased capacity for the oxidation of carbohydrates, the amount of glucose formed from the propionic acid administered was much diminished. The second patient, whose utilization of carbohydrates was much greater, excreted much less glucose as a result of the ingestion of propionic acid.

TABLE I.
Patient J. L.

DATE	CARBO- HYDRATE IN FOOD	NITROGEN	GLUCOSE		G:N	"EXTRA GLUCOSE"	ACETONE	β-HYDROXY- BUTYRIC ACID	
			Gross	Net					
	grams	grams	grams	grams		grams	grams	grams	
Jan.									
19	15.0	12.74	62.7	47.0	3.75				
20	13.1	13.88	62.8	29.7	3.56		4.16*	21.20*	
21	19.6	19.33	91.1	71.5	3.70		4.16*	21.20*	
22	16.0	17.07	99.1	83.1	4.86	20.8	4.00*	22.21*	20.0 gms. pro- pionic acid (equivalent to 24.3 gms. glucose.)
23	15.6	14.61	104.1	88.5	6.06	25.2	4.00*	22.21*	20.0 gms. pro- pionic acid (equivalent to 24.3 gms. glucose.)

The experiment was discontinued because the patient ate other food.

July									
20	17.2	6.93	34.8	17.6	2.53				
21	22.9	8.74	44.5	21.6	2.47				
22	19.6	10.37	56.7	27.1	3.58				
23	17.4	9.23	43.8	26.4	2.86		1.10	7.62	
24	13.0	7.72	58.8	45.8	5.92	23.9	1.43	11.41	36.65 grams propionic acid (equiv- alent to 44.57 grams glucose).
25	19.0	9.85	45.3	26.3	2.69		2.19	14.44	

* Analyses made of two-day composites.

TABLE II.
Patient G. E.

DATE	CARBO- HYDRATE IN FOOD	NITROGEN	GLUCOSE	
	<i>grams</i>	<i>grams</i>	<i>grams</i>	
June				
28	20.5	9.94	26.5	
29	19.3	11.52	26.5	
30	16.1	7.76	11.6	
July				
1	21.0	10.66	38.5	33.6 gms. propionic acid (equivalent to 39.9 gms. glucose).
2	25.5	7.15	18.9	
13	24.2	10.97	27.3	
14	25.2	9.92	26.9	
15	23.9	11.60	28.6	
16	24.9	10.26	37.3	56.8 gms. propionic acid (equivalent to 69.1 gms. glucose).
17	10.2	5.14	27.0	



THE ACTION OF RADIUM EMANATION ON LIPASE.

BY E. K. MARSHALL, JR. AND L. G. ROWNTREE.

(From the Laboratories of Physiological Chemistry and Pharmacology of the Johns Hopkins University.)

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It is claimed that both radium rays and radium emanation possess the power of activating certain enzymes. A certain accelerating influence has been shown for pepsin by the emanation (Bergell and Bickel¹), for autolytic ferments by radium rays (Neuberg,² Wohlgemuth³), and by the emanation (Löwenthal and Edelstein⁴), for tyrosinase by the radium rays (Willecock⁵), for diastase from various origins (Löwenthal and Wohlgemuth⁶), and the uric acid forming ferments of the spleen by the emanation (Schultz⁷). On the other hand, a slight inhibitory effect was observed by Schmidt-Nielsen⁸ for rennin by exposure to strong radium preparations. Trypsin, invertase, and emulsin are reported by Henri and Mayer⁹ to be inactivated through long exposure to the rays.

The possibility of the increased rate of growth in plants¹⁰ (germinating oats, for instance) being due in part to enzymatic activation has been suggested. The idea has been entertained that the therapeutic effect of radium treatment in gout is dependent on this cause. On account of the ease and accuracy of determining quantitatively lipolytic activity, the effect of the radium emanation in this connection has been investigated.

¹ *Verhandl. der Kongr. für inn. Med.*, Wiesbaden, 22d Kongress, 1905, p. 157.

² *Verhandl. d. deutsch. path. Gesellsch.*, vii, p. 157, 1904.

³ *Ibid.*, vii, p. 158, 1904.

⁴ *Biochem. Zeitschr.*, xiv, p. 484, 1908.

⁵ *Journ. of Physiol.*, xxxiv, p. 207, 1906.

⁶ *Biochem. Zeitschr.*, xxi, p. 476, 1909.

⁷ *Ibid.*, xlviii, p. 86, 1913.

⁸ *Mitt. a. Finsen's Med. Lysinst. in Kopenh.*, Jena, 1906, 10 Heft, p. 107.

⁹ *Compt. rend. de l'Acad. des Sci.*, cxl, p. 521, 1904.

¹⁰ Falta and Schwartz: *Berl. klin. Wochenschr.*, xlviii, p. 605, 1911.

The experiments have been carried out with the lipase of pig's liver and the lipase of the castor oil bean. In the case of liver, a 10 per cent "brei" was made, allowed to digest over night, and portions of the clear filtrate used in the experiments either diluted or undiluted. Different liver preparations were used. With the castor bean lipase, the beans were finely ground, extracted with ether in a Soxhlet and weighed portions of this powder used. In some of the experiments with the liver lipase, a saturated aqueous solution of ethyl butyrate was used as substrate, in all other cases 0.5 cc. of ethyl butyrate and 25 cc. of water as the solvent have been used. The mixtures were allowed to digest for a definite time and then titrated with 0.116 N barium hydroxide using 5 or 6 drops of a 1 per cent alcoholic solution of phenolphthalein as indicator. Toluene was used to prevent bacterial growth.

The radioactive solutions were prepared as follows: Radium emanation was collected in the usual way over mercury in a test tube, the amount present being estimated by γ -ray electroscope. This was transferred to a flask containing water, thoroughly shaken for a period of 20 minutes and the amount of emanation per cc. calculated. Varying amounts of this water were added to the lipase-ethyl butyrate mixtures in the amounts indicated.¹¹ The final volume of the mixture was made up to that of the control. The tubes or flasks used were at least half filled with liquid and tightly stoppered with well-fitting corks.

The results of the experiments can be found in the following tables. The activity of the enzyme is expressed in cc. of 0.116 N barium hydroxide necessary to neutralize the butyric acid formed. The values have been in all cases corrected for the acidity developed for the ethyl butyrate and lipase extract alone.

The data of the following tables show conclusively that no accelerating influence is exerted upon the lipase of the pig's liver or castor oil bean by radium emanation in the amounts used. On the contrary, inhibition of the enzymatic activity is suggested.

We acknowledge with pleasure our indebtedness to Dr. H. H. Young, through whose generosity the radium was placed at our disposal.

¹¹ Inasmuch as the flasks were not completely filled with liquid, a large proportion of the radium emanation would eventually be found in the air space above the solution.

TABLE 1.

Ten cc. saturated ethyl butyrate solution, 3 cc. undiluted liver extract, with either 2 cc. water or 2 cc. emanation water (room temperature).

MICROCURIES EMANATION	TIME IN MINUTES	CC. 0.116 N $\text{Ba}(\text{OH})_2$ REQUIRED	
		Without R. E.	With R. E.
46	20	1.96	1.90
4	30	2.35	2.36
18	30		2.40
92	60	2.75	2.80
18	70	3.28	3.30
18	75	3.45	3.35
4	85	3.60	3.50

TABLE 2.

Ten cc. saturated ethyl butyrate solution, 2 cc. undiluted liver extract, with either 1 cc. water or 1 cc. emanation water (room temperature).

TIME IN MINUTES	CC. 0.116 N $\text{Ba}(\text{OH})_2$ REQUIRED	
	Without R. E.	With R. E.
32	1.80	1.76
45	2.30	2.26
83	3.72	3.52
83	3.81	3.50

TABLE 3.

Ten cc. saturated ethyl butyrate solution, 2 cc. undiluted liver extract, 2 cc. water or 2 cc. emanation water.

TIME IN MINUTES	CC. 0.116 N $\text{Ba}(\text{OH})_2$ REQUIRED	
	Without R. E.	With R. E.
25	0.86	0.82
102	1.98	1.65
161	2.20	1.70
161	2.30	2.50
230	2.80	2.07
230	3.00	
284	3.20	2.62

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TABLE 4.

Ten cc. saturated ethyl butyrate solution, 2 cc. undiluted liver extract, and 5 cc. water or 5 cc. emanation water allowed to digest 5 hours at room temperature.

CC. 0.116 N Ba(OH) ₂ REQUIRED	
Without R. E.	With R. E.
3.98	4.22
4.31	4.21
4.16	3.42
4.05	4.13

TABLE 5.

0.5 cc. ethyl butyrate, 1 cc. liver extract (diluted with equal volume of water), and 25 cc. water or 25 cc. emanation water. Allowed to digest 20 hours at 38°.

MICROCURIES EMANATION	CC. 0.116 N Ba(OH) ₂ REQUIRED	
	Without R. E.	With R. E.
17	5.40	4.80
17	6.00	4.85
85	5.70	4.70
85		4.30
0.3		5.95
0.3		5.75

TABLE 6.

0.5 cc. ethyl butyrate, 1 cc. liver extract (diluted with three volumes of water) and 25 cc. water or 25 cc. emanation water digested 44 hours at 38°.

MICROCURIES EMANATION	CC. 0.116 N Ba(OH) ₂ REQUIRED	
	Without R. E.	With R. E.
3	6.70	5.00
3	6.75	5.10
3	6.60	5.10
3	6.60	5.00
0.17	6.75	5.90
0.17		5.80
0.02		6.50
0.02		6.70
0.002		6.70
0.002		6.65

TABLE 7.

0.5 cc. ethyl butyrate, 1 cc. liver extract (diluted three times), 25 cc. water or 25 cc. emanation water. Each flask contained 150 microcuries.

TIME IN HOURS	CC. 0.116 N $\text{Ba}(\text{OH})_2$ REQUIRED	
	Without R. E.	With R. E.
22	4.65	4.30
22	4.80	4.20
48	6.25	5.70
48	6.10	5.80

TABLE 8.

0.5 cc. ethyl butyrate, 0.2 gm. castor bean, and 25 cc. water or 25 cc. emanation water. 25 cc. contained 150 microcuries.

TIME IN HOURS	CC. 0.116 N $\text{Ba}(\text{OH})_2$ REQUIRED	
	Without R. E.	With R. E.
22	1.70	1.65
22	1.65	1.65
48	2.80	2.70
48	2.80	2.70

TABLE 9.

0.5 cc. ethyl butyrate, 0.5 gm. castor bean, and 25 cc. water or 25 cc. emanation water, digested 18 hours at 38°.

MICROCURIES EMANATION	CC. 0.116 N $\text{Ba}(\text{OH})_2$ REQUIRED	
	Without R. E.	With R. E.
3	3.75	3.70
3	3.65	3.65
3	3.75	3.65
150		3.70
150		3.65

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TABLE 10.

0.5 cc. ethyl butyrate, 0.5 gm. castor bean, and 25 cc. water or 25 cc. emanation water, digested 50 hours at 38°. 25 cc. contained 80 microcuries.

CC. 0.116 N $\text{Ba}(\text{OH})_2$ REQUIRED	
Without R. E.	With R. E.
6.35	6.15
6.40	6.15

NOTE ON THE DETERMINATION OF AMINO-ACID NITROGEN IN URINE.

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(Received for publication, October 18, 1913.)

Something over a year ago the writers¹ reported a modified technique for the preparation of urines for the formalin titration method of Henriques and Sørensen. This consisted essentially in the removal of ammonia and other bodies by means of phosphotungstic acid, the clearing-out of the phosphotungstic acid by means of tribasic lead acetate and litharge and the final precipitation of the lead by a stream of hydrogen sulphide. The water-clear filtrate was then neutralized to litmus and titrated to the third stage end-point of phenolphthalein as directed by the original authors.

The results by this method in comparison with those of the "new" method of Henriques and Sørensen² were very much lower. With certain pure substances added to urines values very close to the theoretical were found.

Subsequently the list of pure substances was extended and it was found that a number of amino-acids were removed from the solution at times in whole or in part by the lead. This was notably true of aspartic acid and tyrosine, less so of glutamic acid and leucine. Much depended on the amount of basic lead acetate employed. After a time, however, it became apparent that it would be impracticable to control the quantity of lead and the conditions of temperature, etc., accurately enough to make the method of much value, and a different method of removing the phosphotungstic acid was sought. van Leersum³ had employed KCl for this purpose in the amino-acid method which he devised

¹ Benedict and Murlin: *Proc. Soc. of Exp. Biol. and Med.*, ix, p. 109, 1912.

² *Zeitschr. f. physiol. Chem.*, lxiv, p. 120, 1909.

³ van Leersum: *Biochem. Zeitschr.*, xi, p. 121.

as a modification of the Pfaundler method. The first trials with the method as prescribed by van Leersum were not very successful. Traces of phosphotungstic acid (Merck) could always be found by the zinc test. Later it was found that the 5 per cent KCl solution would remove all of the acid provided a considerable excess of acid was left unprecipitated, and when this was not the case the addition of a little 10 per cent phosphotungstic acid in 2 per cent HCl would bring about complete precipitation. Under these circumstances also KCl could be used in substance and a water-clear filtrate could be readily obtained. Potassium salts however do not remove the phosphates and sulphates; consequently the attainment of an exact neutral point to litmus is very difficult. Going back to barium hydrate as a means of removing the phosphates and sulphates it was found that the barium would suffice also to precipitate the phosphotungstic acid. The whole procedure thus became very simple and as used in these laboratories now is as follows:

PROCEDURE.

1. Measure into a 500-cc. Erlenmeyer flask 200 cc. of a 24-hour human urine diluted to 2000 cc.

2. Add an equal quantity of 10 per cent phosphotungstic acid (Merck⁴) in 2 per cent HCl. Let stand at least three hours; better over night.

3. Pour off 250 cc. of the clear fluid; add 1 cc. of a 0.5 per cent solution of phenolphthalein, and barium hydrate in substance until the whole fluid turns decidedly pink. The barium hydrate should be added a very little at a time. Let stand one hour.

4. Filter off two 100-cc. samples (= 50 cc. urine).

5. Neutralize to litmus (Squibb's papers answer for all practical purposes) with $\frac{N}{8}$ HCl.

6. Add 10-20 cc. neutral formalin and titrate cautiously to deep red color, *i.e.*, until the drop produces no additional color with $\frac{N}{16}$ NaOH.

7. Correct by deducting the amount of $\frac{N}{16}$ NaOH necessary to produce the same depth of color in an equal quantity of CO₂-free water with the same quantity of neutral formalin added.

Some control tests are given below.

⁴ Kahlbaum's preparation is a very different substance.

I. Removal of ammonia by means of phosphotungstic acid.

a. *Pure substances.* 1. 40 cc. $\frac{N}{10}$ solution of aspartic acid + 20 cc. $\frac{N}{10}$ $(\text{NH}_4)_2\text{SO}_4$ solution. 100 cc. 10 per cent phosphotungstic acid solution in 2 per cent HCl added in equal quantity. Stood over night. 5-10 grams KCl added to remove phosphotungstic acid. 100 cc. filtrate titrated 10.1; theory, 10.0.

2. 20 cc. $\frac{N}{10}$ glycocoll + 20 cc. $\frac{N}{10}$ $(\text{NH}_4)_2\text{SO}_4$. 100 cc. Same procedure. 100 cc. filtrate titrated 9.5; theory, 10.0.

b. *Urines.* 1. 200 cc. urine containing 4.6 per cent $\text{NH}_3\text{-N}$ + 200 cc. 10 per cent phosphotungstic acid in 4 per cent HCl stood over night. 20 cc. filtrate aerated by Folin method three hours into 10 cc. $\frac{N}{10}$ H_2SO_4 . Titrated 10.0. Therefore all ammonia out.

2. 100 cc. urine containing 9.5 per cent $\text{NH}_3\text{-N}$ + 100 cc. 10 per cent phosphotungstic acid in 4 per cent HCl. Stood over night. 20 cc. filtrate by Folin method three hours against 10 cc. $\frac{N}{10}$ H_2SO_4 titrated 10.0.

3. 200 cc. urine containing 9.5 per cent $\text{NH}_3\text{-N}$ + 50 cc. 10 per cent phosphotungstic acid in 4 per cent HCl. Stood over night. 20 cc. filtrate by Folin method four hours against 10 cc. $\frac{N}{10}$ H_2SO_4 titrated 6.0. Therefore ammonia not all removed.

4. 100 cc. urine containing 9.4 per cent $\text{NH}_3\text{-N}$ + 50 cc. 10 per cent phosphotungstic acid in 4 per cent HCl. Stood over night. 20 cc. filtrate by Folin method four hours titrated 5.0.

5. 100 cc. urine containing 13 per cent $\text{NH}_3\text{-N}$ + 100 cc. 10 per cent phosphotungstic acid in 4 per cent HCl. Stood one and one-half hours. 20 cc. filtrate by Folin method eighteen hours titrated 9.9.

These results agree with those of Gumlich⁵ in proving that at least an equal quantity of the 10 per cent phosphotungstic acid solution must be added to the urine and that the mixture should stand at least three hours.

II. Removal of phosphotungstic acid by KCl.

1. 100 cc. filtrate from urine (3) above + 100 cc. 5 per cent KCl. Stood two hours. Filtrate gives no blue color with zinc.

2. 100 cc. filtrate from same urine + 10 grams KCl in substance. Stood two hours. Filtrate clear of phosphotungstic acid.

3. a. 200 cc. urine + 200 cc. 10 per cent phosphotungstic acid in 2 per cent H_2SO_4 .

b. 200 cc. same urine + 0.362 gram tyrosine + 200 cc. 10 per cent phosphotungstic acid.

c. 200 cc. same urine + 0.266 gram aspartic acid + 200 cc. 10 per cent phosphotungstic acid.

All stood for one week. Many crystals found on side of flask b and c. Warmed in water bath adding 200 cc. distilled water. Crystals dissolved. Stood four hours. Decanted:

400 cc. clear fluid from each flask + 10 grams KCl. Stood two hours.

⁵ Gumlich: *Zeitschr. f. physiol. Chem.*, xvii, p. 13, 1893.

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100 cc. filtrate from *b* titrated 8.5 cc.; 100 cc. filtrate from *c* titrated 8.5 cc.

100 cc. filtrate from *a* titrated 5.3 cc., 5.3 cc.

Difference $b-a = 3.2$ cc.: $c-a = 3.2$ cc.

Theoretical difference, 3.33 cc.

III. Removal of phosphotungstic acid by $\text{Ba}(\text{OH})_2$.

1. *a.* 200 cc. urine (case of pernicious vomiting) + 200 cc. 10 per cent phosphotungstic acid in 2 per cent HCl.

b. Same containing 0.262 gram leucine. Stood 4 hours. 200 cc. filtrate + 50 cc. saturated solution $\text{Ba}(\text{OH})_2$. Stood one hour.

b. 100 cc. filtrate titrated 6.4 and 6.1 cc. $\frac{N}{10}$ NaOH.

a. 100 cc. filtrate titrated 2.4 and 2.4 cc. $\frac{N}{10}$ NaOH.

Difference, 4.0 and 3.7.

Theoretical difference, 4.0.

2 *a.* 200 cc. urine + 200 cc. phosphotungstic acid.

b. Same containing 0.326 gram tyrosine.

Stood three days. Crystals of tyrosine found on sides of flask. Added few drops concentrated HCl and warmed in water bath until crystals dissolved.

Phosphotungstic removed with $\text{Ba}(\text{OH})_2$ in substance while keeping flask warm.

b. 100 cc. filtrate titrated 9.9 and 10.0.

a. 100 cc. filtrate titrated 4.8 and 4.9.

Difference, 5.1 and 5.1; theory, 5.0.

Aspartic acid added in similar quantity was partially removed by the phosphotungstic acid or possibly by the barium hydrate used in too great concentration.

Levene and Beatty⁶ have shown that while amino-acids in general are not precipitated by phosphotungstic acid unless they are present in great concentration, there is considerable variation in this respect among the individual amino-acids.

Because of the great insolubility of tyrosine, leucine, aspartic acid, etc., in neutral medium, it is important not to let the filtrate stand after the neutral point is reached. The neutral formalin should be added at once.

⁶ Levene and Beatty: *Zeitschr. f. physiol. Chem.*, xlvii, p. 149, 1906.

METABOLISM STUDIES ON COLD-BLOODED ANIMALS. II.

THE BLOOD AND URINE OF FISH.

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(Received for publication, October 20, 1913.)

In a recent paper Folin and Denis¹ have published data regarding the non-protein nitrogen, urea and uric acid in the blood of a number of mammals and a few birds. More or less as a continuation of the work I have collected the blood of a number of the more common fish of the North Atlantic coast and in this blood have determined the total non-protein nitrogen, urea, ammonia, uric acid and creatine.

For the determination of the first four constituents the methods recently published by Folin and Denis² have been used; creatine was determined colorimetrically by the Folin method, the following procedure being employed.

Twenty grams of oxalated blood were poured slowly into 100 cc. of boiling $\frac{N}{100}$ acetic acid solution and the mixture heated for two or three minutes until coagulation was complete; the solution was then filtered, the coagulum returned to the vessel in which the coagulation had been made and washed with about 200 cc. of boiling water. The original filtrate and the wash water were then combined, strongly acidified with acetic acid and rapidly evaporated down to a volume of about 5 cc. This residue was transferred to a small flask, the evaporating dish being first rinsed with 10 cc. of normal hydrochloric acid and then with a few cubic centimeters of water. The mouth of the flask was then loosely closed and the mixture heated for four hours on the boiling water bath. The color was developed in the usual way, but as a standard I used a solution of pure creatinine³ in place of the customary half-normal potassium bichromate.

¹ This *Journal*, xiv, p. 29, 1913.

² *Ibid.*, xi, p. 527, 1912; xiii, p. 469, 1913.

³ *Ibid.*, xii, p. 149, 1912.

The blood was in every case taken from fish, just brought in from the traps and therefore still alive; in the case of the larger specimens the blood was removed from the heart by means of a needle and syringe, while in the case of the smaller animals it was collected from the caudal artery and vein. All figures presented are for whole blood in which coagulation had been prevented by the use of a little solid potassium oxalate. In the case of the larger fish (shark, goosefish, squeteague, etc.) not more than six animals were employed to secure the composite sample of blood, while in the case of the smaller ones (butterfish, mackerel, eel) from fifty to a hundred fish were used in order to obtain the requisite quantity of blood.

Results of the examination of the non-protein nitrogen fraction of the blood of fish.

(The figures represent milligrams per 100 grams of blood.)

	NON-PROTEIN NITROGEN	UREA NITROGEN	AMMONIA NITROGEN	URIC ACID	CREATINE + CREATININE
Dogfish (<i>Mustelis canis</i>).....	1000	800	1.4	0	4.0
Sand shark (<i>Carcharias littoralis</i>).....	1160	1000	2.5	0	4.0
Skate (<i>Raia erniacea</i>).....	1100	868	1.6	0	3.1
Alewife (<i>Ponnolobus pseudoharengus</i>).....	54	10	5.5	1.1	11.0
Butterfish (<i>Poronotus triacanthus</i>).....	50	9	5.1	1.4	16.0
Mackerel (<i>Scomber scombrus</i>).....	86	10	3.8	1.1	7.5
Squeteague (<i>Cynoscion regalis</i>).....	66	20	1.0	0.7	6.0
Menhaden (<i>Brevoortia tyrannus</i>).....	47	10	3.3	1.0	6.0
Summer flounder (<i>Paralichthys dentatus</i>)..	46	8	1.1	0.8	5.0
Shad (<i>Alosa sapidissima</i>).....	90	16		1.1	
Bonito (<i>Sarda sarda</i>).....	90	17	3.8	1.0	
Goosefish (<i>Lophius piscatorius</i>).....	40	8	3.6	0.9	5.0
Eel (<i>Anguilla crysypa</i>).....	50	9	2.8	0.6	10.6

From the figures given in the above table it will be seen that by the new analytical method used I have in the case of the three elasmobranch fishes examined (shark, dogfish and skate) given quantitative confirmation of the earlier observations⁴ regarding the large amount of urea contained in the blood of these animals. In the case of the teleosts, however, the percentage of

⁴ Schröder: *Zeitschr. f. physiol. Chem.*, xiv, p. 576, 1890; Baglioni: *Centralbl. f. Physiol.*, xix, 1905.

non-protein nitrogen accounted for by the urea fraction is much smaller than in the blood of man or of any mammal so far examined. In the series of determinations of urea in the blood of seven different kinds of animals (rabbit, sheep, pig, horse, monkey and beef) recently made with the same method by Folin and Denis;⁵ and in the large number of urea determinations on normal human blood made by the same investigators it was found that the urea nitrogen fraction accounted for about 50 to 60 per cent of the non-protein nitrogen of the blood. In the case of bird blood (chicken, duck and goose) it appears, however, that the urea-nitrogen fraction accounted for only 25 to 30 per cent of the non-protein nitrogen.

The low urea content of bird blood agrees well with what is known regarding the small percentage of the total urinary nitrogen of these animals which is accounted for by urea. Regarding the urinary urea of fish but little is known. In a recent paper⁶ I have reported analyses of the urine of the dogfish in which urea nitrogen amounted to from 80 to 89 per cent of the total nitrogen. The blood of the elasmobranchs differs, however, so markedly from that of the teleosts that it is to be expected that the urine of the two classes would also show marked dissimilarity.

Below is given the result of an examination of a composite sample of the urine of the goosfish (*Lophius piscatorius*). This urine was secured from the bladders of six fish about one hour after death, and was examined on the day of collection. In general it may be said that this urine was a clear, pale yellow fluid, with an acid reaction and a markedly fishy smell. On heating to boiling a heavy coagulum of earthy phosphates was produced which dissolved on the addition of dilute acetic acid.

Analysis of a composite sample of urine from six goosfish.

Specific gravity 1.016. Albumen and reducing sugar absent.

	Mgm. per liter	Per cent of total N		Mgm. per liter
Total N.....	830		Phosphates (as P_2O_5)...	440
Urea N.....	120	14.4	Chlorides (as NaCl).....	10800
Ammonia N.....	12	2.7	Total sulphur.....	108
Uric acid N.....	1	0.1	Inorganic sulphates (as S)	92
Creatinine N.....	7	0.8		
Creatine N.....	140	16.6		

⁵ This *Journal*, xiv, p. 29, 1913.

⁶ *Ibid.*, xiii, p. 225, 1912.

From this examination of the urine of the goosfish, a representative teleost, it is apparent that the small percentage of urea in the blood is also coincident with a small urea excretion in the urine.

The large percentage of undetermined nitrogen in this urine is also noteworthy. It occurred to me that this might be due to the presence in large amounts of amines. Qualitative tests, however, have not confirmed this hypothesis. Another interesting point brought out is the fact that apparently in the urine of a bony fish creatinine is almost entirely replaced by creatine. A somewhat similar condition has been shown to exist in the bird.⁷ It should be remembered, however, that the goosfish is a voracious eater, as much as five pounds of food being frequently found in its stomach and as this food consists of small fish the goosfish must undoubtedly consume a considerable quantity of creatine, a fact which may account in part at least for the large amount of creatine contained in the urine. The high dilution of this urine is also not without interest as it would seem to support the theory that in the fish nitrogenous waste products may be eliminated in part by some organ other than the kidney.

The figures obtained for the ammonia nitrogen fraction of the blood are surprisingly large. A number of ammonia determinations made by the same method by Folin and Denis⁸ showed that the quantity of ammonia in the systemic blood of cats amounts to not more than 0.1 or 0.2 mgm. per 100 grams of blood. Further observations (unpublished) on the ammonia content of normal and pathological human blood have shown that here too ammonia is present to the extent of only a fraction of a milligram per 100 grams of blood.

In connection with the high ammonia content of fish blood it is not inappropriate to mention the well-known experiments of Cohnheim on the deaminizing power of the intestinal mucosa,⁹ in which work, although able to demonstrate the deaminizing power of the surviving intestines of fish, he met with small success when the intestines of cats and dogs were employed.

⁷ Paton: *Journ. of Physiol.*, xxxix, p. 485, 1910.

⁸ This *Journal*, xi, p. 161, 1912.

⁹ *Zeitschr. f. physiol. Chem.*, lix, p. 239, 1909; also lxi, p. 189, 1909.

My results on the uric acid content of the blood and urine of fish are somewhat difficult to explain. As will be noted I have been unable to find more than a minute trace of uric acid in the blood of any of the elasmobranchs examined, while in the blood of all the teleosts it exists in moderate amounts. Uric acid was found in small amounts in the urine of both classes of fish. These findings are contrary to those of Baglioni¹⁰ who states that in the blood of the dogfish there is to be found a larger quantity of uric acid than in the urine.

¹⁰ *Centralbl. f. Physiol.*, xx, p. 105, 1906.



NOTE ON THE TOLERANCE SHOWN BY ELASMOBRANCH FISH TOWARDS CERTAIN NEPHROTOXIC AGENTS.

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Since the initial publications by Schlayer and his associates in 1907 a large number of investigations have been carried out dealing with different phases of the experimental nephritis produced by the injection of various organic and inorganic nephrotoxic agents. In these experiments rabbits, dogs and cats have been the animals invariably used.

In several recent publications¹ it has been shown that in the case of cats and rabbits in whom nephritis had been experimentally produced the non-protein nitrogen of the blood may be increased to many times the normal amount. In these experiments our experience has been that in cases in which the non-protein nitrogen content of the blood was greatly elevated the prognosis was bad.

The elasmobranch fish occupy a unique position with regard to the non-protein nitrogen content of the blood. In these animals this fraction amounts to about 1000 mgm. per 100 grams blood, of which about 80 per cent is urea nitrogen; the tissues likewise contain large amounts of urea, which substance has also been shown to be present in considerable quantities in the intestinal contents, and in the bile.² Elimination by way of the kidneys is small, only 20 to 50 mgm. of urea nitrogen per kilo being excreted in the urine by the starving dogfish in twenty-four hours.³ In view of the above facts it occurred to me that it might be interesting to see whether the elasmobranch fishes might not be

¹ Folin, Karsner and Denis: *Journ. of Exp. Med.*, xvi, p. 789, 1912; Frothingham, Fitz, Folin and Denis: *Arch. of Int. Med.*, xii, p. 245, 1913.

² Van Slyke and White: this *Journal*, ix, p. 209, 1911.

³ Denis: *ibid.*, xiii, p. 225, 1912.

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able to withstand the administration of relatively enormous doses of renal poisons.

For all experiments the smooth dogfish (*Mustelis canis*) was used. During the experiment the animals were kept in large tanks supplied with running sea water and were fed every second day with fish; in many cases, however, food was refused.

As the number of tanks available was somewhat limited I have confined myself to the study of two well-known nephrotoxic agents, *i.e.*, uranium nitrate and potassium chromate. These substances were administered by means of subcutaneous injections into the flank, each dose being injected into several different places.

As will be seen by inspection of Table I, uranium nitrate may be administered to the dogfish in doses as large as 80 mgm.⁴

TABLE I.
Uranium nitrate experiments.

NUMBER OF FISH	WEIGHT IN GRAMS	URANIUM NITRATE GIVEN, AS MILLIGRAMS PER KILO	REMARKS
1	1010	64	Killed 5 days after injection.
2	1015	64	Killed 5 days after injection.
3	2050	32	Killed 5 days after injection.
4	1012	64	Killed 5 days after injection.
5	1500	53	Killed 5 days after injection.
6	1500	53	Killed 5 days after injection.
7	1020	80	Killed 5 days after injection.
8	800	100	Killed 5 days after injection.
24	1350	60	Killed 5 days after injection.
31	700	80	Killed 6 days after injection.
32	1550	51	Killed 7 days after injection.
62	1000	80	Killed 6 days after injection.
63	1200	66	Killed 6 days after injection.
64	1350	60	Killed 8 days after injection.
65	1550	50	Killed 8 days after injection.
23	750	106	Died 68 hours after injection.
27	630	126	Died 26 hours after injection.
29	570	140	Died 43 hours after injection.
28	510	156	Died 37 hours after injection.

⁴ Rabbits are frequently rendered anuric by doses of 1 to 3 mgm. of uranium nitrate per kilo of body weight; dogs and cats are somewhat less sensitive, but even with these animals serious symptoms are obtained by the administration of 5 to 10 mgm. per kilo.

per kilo of body weight without ill effects. All animals killed remained in excellent condition during the entire experimental period.

A similar series of experiments was undertaken in which potassium chromate was administered. The results are given in the following table, and show that here again the dogfish appears to be very resistant towards this class of poisons.

TABLE II.
Potassium chromate experiments.

NUMBER OF FISH	WEIGHT IN GRAMS	POTASSIUM CHROMATE GIVEN AS MILLIGRAMS PER KILO	REMARKS
37	1350	48	Killed 5 days after injection.
38	1150	55	Killed 6 days after injection.
39	1080	59	Killed 6 days after injection.
40	1090	59	Killed 6 days after injection.
19	1020	94	Killed 5 days after injection.
20	1080	88	Killed 5 days after injection.
18	600	166	Died 40 hours after injection.

An attempt was also made in a few cases to determine whether the accumulation of nitrogenous waste products could be demonstrated in dogfish in whom nephritis had been experimentally induced. In order to obtain an idea of the average amounts of total non-protein nitrogen and urea nitrogen present in the blood of the dogfish, samples of blood were secured from twenty different animals, care being taken to choose fish of varying sex, weight and age. In these samples urea and total non-protein nitrogen were determined by the methods of Folin and Denis.⁵ The maximum, minimum and average values found were as follows (results are expressed as milligrams per 100 grams of blood):

	Maximum	Minimum	Average
Non-protein nitrogen.....	1240	900	1000
Urea nitrogen.....	960	713	800

As will be seen by the results presented in Table III no accumulation could be demonstrated in dogfish to whom large doses of uranium nitrate and potassium chromate had been given, a

⁵ This *Journal*, xi, p. 527, 1912.

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TABLE III.

Non-protein nitrogen and urea in the blood of nephritic dogfish.
(Milligrams per 100 grams blood.)

LABORATORY NUMBER OF FISH	UREA NITROGEN	TOTAL NON-PROTEIN NITROGEN	LABORATORY NUMBER OF FISH	UREA NITROGEN	TOTAL NON-PROTEIN NITROGEN
2	757	1000	32	765	990
3	800	1050	28	713	900
4	840	1120	19	687	875
8	880	1250	39	713	925
31	800	1000	40	687	920

result not surprising if we take into consideration the small elimination by the kidneys, and the apparent ability of these animals to utilize the liver and perhaps the intestine as an excretory organ.

An attempt was made with a number of animals to collect samples of urine by means of a cannula tied in the urinary papilla; in every case, however, anuria had apparently set in by the third or fourth day so that I am unable to report the results of any urine examinations.

UREA FORMATION IN THE LIVER.¹

A STUDY OF THE UREA-FORMING FUNCTION BY PERFUSION WITH FLUIDS CONTAINING (a) AMMONIUM CARBONATE AND (b) GLYCOCOLL.

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INTRODUCTION.

It is a desirable thing to be able to localize special functions to definite sites. Attempts of this sort are frequently made, and in a large number of cases it has been the liver to which attention has been directed. There seem to be a variety of reasons for this. One is that the location of this organ is such as to suggest that it serves the function of protecting the organism in general from toxic substances entering the circulation from the digestive tract; another, that a considerable amount of evidence of such a protective function exists; still another, that its size is taken, and probably rightly, to be more or less of an index of its importance. Undoubtedly it has often served as the scapegoat where ignorance has existed, especially from a clinical standpoint. Perhaps, also, the fact that the liver is a comparatively easy organ to perfuse by itself has something to do with its popularity. That its importance as a specific site of metabolic processes has been overestimated is shown by the decrease in the rôle ascribed to it in carbohydrate metabolism in past years. Fischler and Bardach² have apparently demonstrated that normal utilization of sugar can take place when it is severely injured.

Among the processes at various times attributed to the liver, few have been so prominent and so much discussed as that of the formation of urea, especially from ammonium salts and from amino-acids.

¹ Aided by a grant from the Rockefeller Institute for Medical Research.

² *Zeitschr. f. physiol. Chem.*, lxxviii, p. 435, 1912.

Concerning the ability of the liver to synthesize urea from ammonium salts of organic acids which are oxidized in the body to carbon dioxide and water, there can be no doubt. The first demonstration of this by v. Schroeder,³ including the isolation and identification of urea nitrate, took place and was confirmed long ago. The purpose of such a function appears definite in the light of recent investigations by Folin and Denis,⁴ which demonstrate that the ammonia of the portal blood originates largely in the large intestine and is therefore chiefly a bacterial product. It is easy to understand why it should be rendered innocuous before being distributed to the tissues. That the liver is the sole site of this process is far from being a proved fact. The old assumption that the increase in the excretion of ammonia in disturbances of the liver is due directly to hepatic insufficiency need not be considered, since the evidence against it is too great (Muenzer⁵ and others).

Quite otherwise, however, stand the facts concerning urea formation from amino-acids. According to the view which appeared most probable until recently, the amino-acids are deaminized somewhere between the lumen of the intestine and the liver, the nitrogen being carried in the form of ammonia to the liver, there to be converted into urea, or else being immediately resynthesized into protein in the intestinal wall. Various discoveries by Folin and Denis⁶ have greatly modified the status of this question: (1) the demonstration of the rapid distribution of amino-acids *as such* to the tissues, *without* immediate alteration, the storage of the same in a "nitrogen reservoir" (at least partly in the muscles) and their later conversion into urea, and (2) the finding of ammonia in the portal (and systemic) blood in concentrations much below those previously supposed to exist, with the major part of it coming from the large intestine. These findings make it quite unnecessary to assume that either deamination or resynthesis occurs in the intestinal wall.

The only direct evidence now existing of the occurrence of urea

³ *Arch. f. exp. Path. u. Pharm.*, xv, p. 364, 1882.

⁴ *This Journal*, xi, p. 161, 1912.

⁵ *Deutsch Arch. f. klin. Med.*, lii, p. 199, 1894; *Arch. f. exp. Path. u. Pharm.*, xxxiii, p. 164, 1894.

⁶ *This Journal*, xi, pp. 87, 161, 1912; xii, p. 141, 1912.

formation directly from amino-acids in the liver is that of Salaskin,⁷ which has stood for fifteen years with no great amount of criticism except to the effect that he did not isolate urea. His results, obtained by the perfusion of the liver with amino-acids, which showed an increase in urea by the method of Schöndorff, can hardly be accepted. In the first place he gives only the percentage of urea (amid-nitrogen) in the perfusing fluid, without stating the amount of fluid recovered. It is therefore impossible to know how much urea (total amount) there really was in the fluid at the end of any experiment, although with the amounts used (about 1000 cc.) it is not likely that any large proportion of the volume disappeared by concentration. The point in his results that stands out most prominently, however, is the fact that in all but one of four experiments in which he made analyses in the middle of the perfusion (including both of the two with glycocoll) he obtained a *greater increase in urea in the second than in the first half of the experiment* (lasting about three or four hours). It is quite inconceivable that such a result could be obtained with an organ removed from the body in an experiment begun fifteen to thirty-five minutes after the death of the animal and lasting more than three hours. If he did actually find such an increase in urea as his results indicate, a certain amount of it at least can be explained only as a result of abnormal post-mortem change. The liver certainly could not be more active in the second half of the experiment than in the first. The possibility suggests itself that ammonia was formed from amino-acids by autolysis or bacteria, or both. Any decisive information for or against such a possibility need hardly be attempted now, inasmuch as no one knows the nature, the abundance or the activity of the flora inhabiting his material, or the relationships of factors governing autolysis therein. If the above suggestion be the correct one, the increase in urea is easily explained. Certain it is that a liver, after a three-hour perfusion, is far from normal in appearance (edema, hemorrhages, excessive fragility, etc.), so that degenerative changes are quite conceivable. That ammonia is formed in the course of a comparatively short perfusion is evident from our results below. The assumption of such a formation of ammo-

⁷ *Zeitschr. f. physiol. Chem.*, xxv, p. 128, 1898.

nia in the experiments of Salaskin might possibly explain his failure to get any evidence of urea formation in the perfusion of muscle, inasmuch as urea formation from ammonia has never been demonstrated there, although that it may occur is still quite possible. Since Salaskin's figures cannot be interpreted as meaning that all the increase in the urea values found represents urea (amid-nitrogen) formation from amino-acids, it is impossible to prove that any of it does.

Another possible explanation of the discrepancy between the results of Salaskin and the quite different ones obtained by us (see below) lies in a consideration of the methods employed. It is interesting to note that analyses of samples of the same material (intestinal mucosa) by Salaskin and Kowalewsky,⁸ using the Schöndorff and the Mörner-Folin methods (applied to tissue analysis) showed 32 mgms. per 100 grams by the former, and only 14 mgms. by the latter, whereas on the other hand, urea determinations by the Schöndorff method for urine tend to give lower results (Folin⁹). Furthermore, the normal urea-content of human blood (expressed as nitrogen) by the Schöndorff method has been found to be 23 to 28 mgms. per 100 cc. by v. Jaksch¹⁰ (Schöndorff¹¹ earlier, in one case, found 28.5 mgms.), while by the method of Folin and Denis¹² it is uniformly 11 to 13 mgms. per 100 cc. The figures of v. Jaksch and of Schöndorff for the urea nitrogen are practically the same as those obtained by Folin and Denis for the total non-protein nitrogen (22 to 26 mgms. per 100 cc.). Therefore, it is extremely probable that the Schöndorff figures include something not hydrolyzed by the method of Folin and Denis. It would appear that this substance, whatever it may be, is present in relatively greater concentration in the blood than in the urine.

Leaving out of consideration experiments with organ extracts and the like *in vitro* (Jacoby, Gottlieb, Lang and others), which, as suggested by Folin and Denis,¹³ are quite unacceptable (since

⁸ *Zeitschr. f. physiol. Chem.*, xlii, p. 410, 1904.

⁹ *Ibid.*, xxxii, p. 504, 1901.

¹⁰ *Internat. Beitr. z. inn. Med.*, i, p. 197, 1902. Quoted by Maly, xxxii, p. 265, 1902.

¹¹ *Pflüger's Archiv*, lxxiv, p. 307, 1899.

¹² *This Journal*, xiv, p. 29, 1913.

¹³ *Ibid.*, xi, p. 527, 1912.

the abnormal production of ammonia first is not excluded), the only other evidence of urea formation from amino-acids in the liver has been derived from the results of operations upon the liver (Eck fistula, extirpation, etc.) and from liver disease (in human beings, and experimentally in animals). In the former case the evidence is based on urine analyses made either shortly after the operation, or later during a period of acute intoxication as a result of feeding meat, etc.; in either event the animal is distinctly abnormal (not solely with respect to its liver) and we believe that conclusions drawn from such results have no importance whatsoever as bearing upon this question, for it is certainly impossible to confine a general acute intoxication to a single organ. For instance, it has frequently been entirely disregarded that many of the procedures used in such experiments produce a disturbance of renal activity, and no one knows how great a part the nephritic element has played in such results, for it is now well known that the absence of albumen and casts is far from being an absolute proof of normal renal function.

As far as urine analyses in liver disease are concerned, Fawitzky¹⁴ demonstrated nearly a quarter of a century ago that the chief cause of the earlier obtained low urea values was purely a result of the low protein intake. When the true significance of the increased excretion of ammonia in such conditions was brought to light (Muenzer¹⁵), the interest attached to this product was transferred to the amino-acids.

The evidence from the examination of the urine in cases of liver disease, in recent times, has consisted chiefly in demonstrations, by methods of very varying degrees of accuracy, of the existence of an increased excretion of amino-acid nitrogen, and of the recovery of amino-acids from the urine after they have been fed in amounts supposed to be largely destroyed by the normal individual (the latter primarily by Glaessner¹⁶). In the first place it is quite impossible to say, in any case of liver disease, that the liver is the sole site of functional disturbance, and therefore, no matter how important such findings may prove to be for the clinical diagnosis of such affections, it is not permissible to draw definite conclusions

¹⁴ *Deutsch. Arch. f. klin. Med.*, xlv, p. 429, 1889.

¹⁵ *Loc. cit.*

¹⁶ *Zeitschr. f. exp. Path. u. Ther.*, iv, p. 336, 1907.

as to the normal functions of the liver from urinary findings in disease-complexes in which the liver merely dominates the clinical and the anatomical pictures. The assumption that the increased excretion of amino-acids in cirrhosis, for example, is due in part at least to autolysis is supported by Samuely's¹⁷ finding of a similar phenomenon in lobar pneumonia at the time of the crisis (isolation of abnormally large amounts of β -naphthalinsulphoglycine), although before this result appeared it was stated that the absence of such an increase in pneumonia was against the view that autolysis was concerned. As a matter of fact, the results of investigations of this nature in liver disease so far have been exceedingly variable. For example, Bergell and Blumenthal¹⁸ found a normal nitrogen partition and normal behavior of 20 grams of alanine in acute yellow atrophy. Masuda¹⁹ also has obtained results quite different from those of Glaessner. Certainly there is no functional liver test that gives results sufficiently constant for definite conclusions to be based on them, especially since the conditions in which they are the most constant are acute toxic states (when organs other than the liver are similarly damaged) and advanced stages of more or less localized, extensively destructive diseases of the liver (in which the patient is far from being functionally normal otherwise than as to his liver). Even in the most extensive of these, viz., acute yellow atrophy and phosphorus poisoning, a number of cases have been reported in which normal amounts of urea and other nitrogenous constituents (allowing when necessary for "neutralization ammonia") were excreted, and in some of these instances amino-acids administered were destroyed to a normal extent (Rosenheim,²⁰ Muenzer,²¹ Badt,²² Richter,²³ Neuberg and Richter,²⁴ Bergell and Blumenthal,²⁵ Ishihara²⁶ and others). It is evident, therefore, from the numerous inconsisten-

¹⁷ *Zeitschr. f. physiol. Chem.*, xlvii, p. 377, 1906.

¹⁸ *Charité-Annalen*, xxx, p. 19, 1906.

¹⁹ *Zeitschr. f. exp. Path. u. Ther.*, viii, p. 629, 1911.

²⁰ *Zeitschr. f. klin. Med.*, xv, p. 441, 1888.

²¹ *Loc. cit.*

²² *Centralbl. f. klin. Med.*, xiii, p. 251, 1891.

²³ *Berl. klin. Wochenschr.*, 1896, p. 454.

²⁴ *Deutsch. med. Wochenschr.*, 1904, p. 499.

²⁵ *Loc. cit.*

²⁶ *Biochem. Zeitschr.*, xli, p. 315, 1912.

cies in the literature, that the time is not yet when the nature of normal processes occurring in the liver can be definitely settled by the examination of the urine in cases of liver disease. Such investigations will be of more value when they can be carried on from another view-point, for when the physiology of the liver as learned by more direct methods shall become better understood, their results may be of importance in determining more nearly what actually occurs in such disturbances.

On the other side, an important piece of evidence in favor of the assumption that the liver is not a special site of urea formation from amino-acids has appeared in the work of Folin and Denis,²⁷ in which they have failed to find, by a method which as they say could hardly fail to show it if it existed, any difference in the urea-content of the hepatic venous blood and of blood from other parts of the body while non-protein nitrogen from amino-acids or Witte's peptone was being absorbed from the small intestine and the urea content of the blood increasing.

EXPERIMENTAL.

Our experiments consist in the perfusion of the livers of rabbits and cats with defibrinated blood, with or without the addition of serum and Ringer's solution or of the latter alone. The methods used have permitted the employment of comparatively small quantities of fluid (usually about 100 cc.). In all the cat experiments the blood has been that of the animal whose liver was used; in all, the fluid has been from the same species. We have performed experiments of two kinds; with the addition of (1) commercial ammonium carbonate, and (2) glyccoll, to the perfusing fluid.

Method.

The analyses were made in duplicate. Total non-protein nitrogen, urea and ammonia were all determined by the recently devised methods of Folin and Denis.²⁸ The figures given as urea nitrogen represent, of course, everything hydrolyzed in a constant boiling mixture consisting chiefly of potassium acetate at about

²⁷ This *Journal*, xii, p. 141, 1912.

²⁸ *Ibid.*, xi, p. 527, 1912.

150°C. in ten minutes, minus the ammonia nitrogen separately determined.

Technically, the experiments were arranged so that small quantities of perfusing fluid could be used, passing continuously and repeatedly in the same direction at an approximately constant temperature. The fluid was accurately measured at the beginning and end of the experiment and was usually about 100 cc. The temperature was usually at 38°C., but occasionally momentarily rose to as much as 40° and very rarely sank to 35°. The accompanying diagram shows the scheme adopted for the use of small quantities of fluid, the bottles being of 125 cc. capacity with wide necks. Perforated rubber stoppers were used and rubber tubing of 2 mm. caliber. The fluid passed out of one bottle through the liver and into the other bottle, then by throwing over the rocker valve, the filled bottle was made the supply bottle, the fluid continuing to flow in the same direction without noticeable interruption. The tube conducting fluid away from the bottles was connected with a coil of glass tubing in a leaden box containing water heated by an electric stove. From the coil the fluid passed to a T-tube in which the bulb of a thermometer was placed so that one arm connected with the heating coil, one arm with the thermometer, and one arm with a rubber tube 10 cm. in length which led to a cannula in the portal vein. The tubing from the T-tube to the portal vein, as well as the liver, trunk of the animal and several centimeters of tubing leading away from the inferior vena cava were contained in a double walled tin box, in which a temperature of about 40°C. was maintained by means of an electric light bulb.

All animals were bled to death before using the liver for perfusion. In the case of the cat, the animal was etherized and bled from a cannula placed in the carotid artery. In this way sufficient blood could be collected from one animal to serve for the experiment with its own liver. The blood was rapidly defibrinated in a flask with glass beads and the blood mixed with the other materials as described above. As soon as bled, the abdomen and thorax were opened, glass cannulae placed in the portal vein and in the inferior vena cava immediately above the diaphragm. The aorta and vena cava were ligated beneath the diaphragm and the

lower part of the trunk and upper part of the thorax severed. The remaining part of the trunk including liver and intestines was wrapped in towels soaked in hot salt solution (0.85 per cent NaCl), placed in the tin box and connected with the tubing so that the perfusing fluids entered the portal vein. This procedure occupied from ten to twenty minutes.

In the case of the rabbit, the same technique was used except that usually the blood of two animals had to be mixed in order to obtain the proper amounts. These animals were bled by a rapid severing of the femoral artery so that a minimum of ether was used. The animals were dead before being opened for exposure of the liver.

The length of time occupied in the perfusion was in most instances about one hour. The definite results obtained with ammonium carbonate in this length of time, combined with the distinctly abnormal appearance of the liver after several hours' perfusion and the incongruous results obtained by Salaskin appear to justify the employment of short experiments. In every case the blood was removed from the liver by previously perfusing with Ringer's solution, therefore it was not considered necessary to run the fluid through before making the first analysis. The substance used in each case was added in aqueous solution to the fluid and the two thoroughly mixed by shaking. Samples were then taken for analysis (5 cc. for precipitation with acetone-free methyl alcohol, 4 to 10 cc. for duplicate ammonia analyses) and the fluid poured into one of the bottles. Samples were again taken at the end of the experiment (and in one case during its course). The total quantities of the various substances analyzed were calculated from the amounts of fluid put in at the beginning and removed at the end of each experiment in those cases in which obvious loss, as a result of leakage, etc., did not occur. The remarkable constancy of the total non-protein nitrogen in most of the experiments in which there was no such loss (Experiments 10 and 11 are exceptions) indicates how slight must have been any washing out from or absorption by the liver during their course. Naturally the fluid left in the liver could not be added to that recovered, nor was its amount determined, but there can be no doubt that calculations based upon the total amount recov-

ered are of more value than mere percentages. Slowtzo²⁹ and Ssobilew²⁹ furthermore found only 2.0 to 5.6 per cent of blood in the livers of human cadavers by colorimetric determination.

I. Ammonium carbonate.

EXPERIMENT 1. Normal rabbit. Fluid: 23 cc. rabbit serum, 36 cc. defibrinated rabbit blood, 31 cc. Ringer's solution and 101 mgms. of commercial ammonium carbonate. Sixty-five minutes, nineteen times. In, 100 cc. Out, 115 cc.

	MILLIGRAMS PER 100 CC.		TOTAL AMOUNT IN MILLIGRAMS	
	Total non-protein nitrogen	Ammonia nitrogen	Total non-protein nitrogen	Ammonia nitrogen
Before.....	62	22	62	22
After.....	54	7	62	8

EXPERIMENT 2. Normal rabbit. Fluid: 23 cc. rabbit serum, 20 cc. defibrinated rabbit blood, 57 cc. Ringer's solution and 103 mgms. ammonium carbonate. One hour, forty times. In, 100 cc. Out, 98 cc. (diluted to 155 cc.).

	MILLIGRAMS PER 100 CC.		TOTAL AMOUNT IN MILLIGRAMS	
	Total non-protein nitrogen	Ammonia nitrogen	Total non-protein nitrogen	Ammonia nitrogen
Before.....	66	24	66	24
After.....	43	5	67	8

In the following three experiments (3, 4 and 5) accidents caused the loss of considerable amounts of fluid, therefore the total recovery is unknown.

EXPERIMENT 3. Normal rabbit. Fluid: 18 cc. rabbit serum, 53 cc. defibrinated rabbit blood, 9 cc. Ringer's solution, 20 cc. normal saline solution, 71 mgms. glyecoll and 45 mgms. ammonium carbonate. In, 100 cc.

	MILLIGRAMS PER 100 CC.	
	Total non-protein nitrogen	Ammonia nitrogen
Before.....	62	14
After.....	60	4

²⁹ *Biochem. Zeitschr.*, xxxi, p. 234, 1911.

EXPERIMENT 4. Liver of rabbit in which biliary cirrhosis had been produced by ligation of the common bile-duct five weeks before (Richardson³⁰). Fluid: 63 cc. defibrinated rabbit blood, 37 cc. Ringer's solution and 104 mgms. ammonium carbonate. Thirty-two minutes, five times. In, 100 cc.

	MILLIGRAMS PER 100 CC.			
	Total non-protein nitrogen	Urea nitrogen	Ammonia nitrogen	Rest nitrogen
Blood.....	43	18	Trace ³¹	25
Fluid before.....	51	11 ³²	24	16
Fluid after.....	62	26	7	29

The ammonia fell from 47 to 11 per cent of the total non-protein nitrogen (decrease of 77 per cent.) The urea increased from 22 to 42 per cent of the total non-protein nitrogen (an increase corresponding to 56 per cent of ammonia lost). Although a perfectly definite increase in urea (amid nitrogen) is seen, 44 per cent of the lost ammonia was not recovered as urea. The two possibilities are: (1) that the remainder of the ammonia was retained in the liver, and (2) that it was converted into something other than urea. The results of Experiment 6 throw some light upon this.

EXPERIMENT 5. Normal rabbit. Starved forty-eight hours. Fluid: 65 cc. defibrinated rabbit blood, 35 cc. Ringer's solution and 105 mgms. ammonium carbonate. Twenty-eight minutes, eleven times. In, 100 cc.

	MILLIGRAMS PER 100 CC.	
	Total non-protein nitrogen	Ammonia nitrogen
Before.....	54	21
After.....	60	7

³⁰ *Journ. of Exp. Med.*, xiv, p. 401, 1911.

³¹ Quantitative ammonia determinations were not made when 10 cc. of the Nesslerized solution could not be read in the ordinary colorimeter. Where a "trace" is reported, however, it is certain that there was considerably less than 0.5 mgm. in 100 cc. of the blood. This and other analyses of normal blood from both rabbits and cats confirm the findings of Folin and Denis, viz., that the ammonia in the systemic blood of normal animals is present only in traces.

³² Calculated from the amount found in the defibrinated blood before anything was added to it, on the basis of the dilution.

EXPERIMENT 6. Normal cat. Starved twenty-four hours. Fluid: 40 cc. defibrinated cat blood, 35 cc. Ringer's solution and 107 mgms. ammonium carbonate dissolved in 4 cc. water. One hour, forty-five times. In, 80 cc. Out, 92 cc.

	MILLIGRAMS PER 100 CC.				TOTAL AMOUNT IN MILLIGRAMS			
	Total n.p. nitrogen	Urea N	Ammonia N	Rest N	Total n.p. nitrogen	Urea N ²³	Ammonia N	Rest N
Blood.....	51	23	Trace	31	20	9	Trace	11
Fluid before.....	52		28		42		23	
Fluid after	48	15	7	26	44	14	6	24

The last experiment, in which a complete recovery was made, shows the same thing as Experiment 4. Seventy-two per cent of the ammonia disappeared, but only 26 per cent of the lost ammonia was recovered as urea. The constancy of the total non-protein nitrogen here, as in other experiments in which practically everything was recovered, is greatly against the assumption that any appreciable amount of nitrogen, in the form of ammonia or otherwise, has been retained in the liver. The conclusion of Kowalewsky and Markewicz²⁴ from their perfusion experiments, that the ammonia is deposited in the perfused organ, is based upon two experiments with muscle, in which increases of 4.8 and 7.8 mgms. of ammonia per 100 grams, respectively, were found, after perfusion with blood containing about 14 mgms. per 100 grams; the method (distillation with magnesia) is certainly not accurate for blood, and is therefore even less likely to be so for tissues. If the in-

²³ The determination of urea in the presence of comparatively large amounts of ammonia by the method used has not given satisfactory results, the loss occurring, as far as we now know, in evaporating off the methyl alcohol. For this reason the urea has been determined in Experiments 4 and 6 in the blood before adding the ammonium carbonate, and the urea content of the fluid calculated from the result. For the same reason, the figures for the urea at the end of the experiment are in all probability minimal, for it is very likely that some ammonia is lost even there. Even assuming, however, that the total amount of nitrogen obtained after hydrolysis in determining the urea and ammonia together represents urea nitrogen alone, which cannot be so, for with the larger amounts the loss was only about 20 per cent, there is still not enough increase to equalize the loss in ammonia during the perfusion.

²⁴ *Loc. cit.*

crease in "rest nitrogen" in our experiments were due to washing out from the liver, it is hard to see why it should so nearly equal the ammonia unaccounted for.

In another experiment with ammonium carbonate, all but the ammonia determinations miscarried.

EXPERIMENT 7. Normal cat. Fluid: 87 cc. defibrinated cat blood and 103 mgms. ammonium carbonate in 5 cc. Ringer's solution. Perfused one hour.

	MILLIGRAMS PER 100 CC.	TOTAL AMOUNT IN MILLIGRAMS
	Ammonia nitrogen	Ammonia nitrogen
Before.....	23	21
After.....	7	7

The results of all the experiments with ammonium carbonate are given below in tabular form:

MILLIGRAMS PER 100 CC.

NUMBER OF EXP.	Before				After			
	Total n.p. nitrogen	Urea N	Ammonia N	Rest N	Total n.p. nitrogen	Urea N	Ammonia N	Rest N
1	62		22		54		7	
2	66		24		43		5	
3	62		14		60		4	
4	51	11	24	16	62	26	7	29
5	54		21		60		7	
6	52	11	28	13	48	15	7	26
7			23				7	

NUMBER OF EXP.	AMMONIA N IN PER CENT OF TOTAL N.P. N		UREA N IN PER CENT OF TOTAL N.P. N		PER CENT OF AMMONIA N LOST	PER CENT OF LOST AMMONIA N RECOVERED AS UREA
	Before	After	Before	After		
1	35	13			63	
2	36	12			67	
3	23	7			70	
4	47	11	22	42	77	56
5	39	12			69	
6	54	15	21	31	72	26

Therefore, from 63 to 77 per cent of the added ammonia disappeared, and of this only a part was recovered as urea.

II. *Glycocoll*.³⁵

EXPERIMENT 8. Normal rabbit. Fluid: 46 cc. rabbit serum, 36 cc. defibrinated rabbit blood, 23 cc. Ringer's solution and 183.8 mgms. glycocoll (34.3 mgms. nitrogen). Fifty minutes, twenty times. In, 105 cc. Out, 102 cc.

	MILLIGRAMS PER 100 CC.		TOTAL AMOUNT IN MILLIGRAMS	
	Total non-protein nitrogen	Urea and ammonia nitrogen	Total non-protein nitrogen	Urea and ammonia N.
Before.....	76	20	80	.21
After.....	76	19	77	19

EXPERIMENT 9. Normal rabbit. Fluid: 31 cc. rabbit serum, 54 cc. defibrinated rabbit blood, 15 cc. Ringer's solution and 122.3 mgms. glycocoll (22.8 mgms. nitrogen). Sixty-one minutes. Thirty times. In, 100 cc. Out, 115 cc.

	MILLIGRAMS PER 100 CC.		TOTAL AMOUNT IN MILLIGRAMS	
	Total non-protein nitrogen	Urea and ammonia nitrogen	Total non-protein nitrogen	Urea and ammonia N.
Before.....	70	22	70	22
After.....	60	17	69	20

In the next two experiments, with cats, there appears to have been a certain amount of nitrogen washed out of the liver.

EXPERIMENT 10. Normal cat. Liver somewhat fatty. Starved twenty-four hours after meat régime. Fluid: 44.5 cc. defibrinated cat blood, 58 cc. Ringer's solution and 188.5 mgms. glycocoll (35.2 mgms. nitrogen). One hour, eighteen times. In, 102 cc. Out, 122 cc.

	MILLIGRAMS PER 100 CC.		TOTAL AMOUNT IN MILLIGRAMS	
	Total non-protein nitrogen	Urea and ammonia nitrogen	Total non-protein nitrogen	Urea and ammonia N.
Before.....	62	10	63	10
After.....	65	9	79	11

³⁵ Kahlbaum's glycocoll was used in all cases.

EXPERIMENT 11. Normal cat. Fluid: 56 cc. defibrinated cat blood, 19 cc. Ringer's solution and 188.7 mgms. glycocoll (35.2 mgms. nitrogen) in 5 cc. water. One hour. Thirty-one times. In, 80 cc. Out, 105 cc.

	MILLIGRAMS PER 100 CC.		TOTAL AMOUNT IN MILLIGRAMS	
	Total non-protein nitrogen	Urea and ammonia nitrogen	Total non-protein nitrogen	Urea and ammonia N.
Before.....	72	17	58	14
After.....	66	15	69	15

In the fluid at the end of the perfusion were found 1.9 milligrams of ammonia nitrogen, making the total urea nitrogen only 13 mgm. A comparison of this result with the normal amount of ammonia nitrogen in cat's blood found by Folin and Denis (about 0.07 mgm. per 100 cc.) demonstrated that there is formation of ammonia during the course of an hour's perfusion. The next experiment (12) shows the same thing. As stated earlier, in no case have we been able to get enough ammonia from 5 cc. of normal blood to be anywhere nearly readable in the Duboscq colorimeter as ordinarily used, *i.e.*, without the polariscope tube and iris diaphragm used by Folin and Denis.

EXPERIMENT 12 Normal cat. Starved twenty-four hours. Fluid: 76 cc. blood and 179.5 mgms. glycocoll (33.5 mgms. nitrogen) in 4 cc. water. Total time, three hours. Analysis of fluid after one hour as well as at end. In, 80 cc. Out, in one hour, 105 cc.: in, three hours, 84 cc.

	MILLIGRAMS PER 100 CC.				TOTAL AMOUNT IN MILLIGRAMS			
	Total n.p. N	Urea and amm. N	Urea N	Ammonia N	Total n.p. N	Urea and amm. N	Urea N	Ammonia N
Before.....	75	12.0			60	9.6		
After 1 hour..	58	10.0			{ removed 61	10.5		
					{ returned 58	10.0		
After 3 hours.	69	13.8	11.6	2.2	58	11.6	9.8	1.8

5 cc. of the 105 removed at the end of one hour were used for analysis, the other 100 cc. returned to the apparatus.

In the following experiment the outflow of fluid from the liver was obstructed early in the perfusion, causing the liver to swell permanently. Therefore, the recovery was only partial. Ringer's

solution had to be added to make the volume large enough to continue the experiment.

EXPERIMENT 13. Normal cat. Starved twenty-four hours after meat diet. Fluid: 81 cc. defibrinated cat blood and 173 mgms. glycocoll (32.3 mgms. nitrogen) in 9 cc. water. Forty minutes, thirty times. In, 90 cc. Out, 97 cc.

	MILLIGRAMS PER 100 CC.		TOTAL AMOUNT IN MILLIGRAMS		$\frac{\text{UREA} + \text{NH}_3\text{-N}}{\text{TOTAL N. P. N}}$
	Total non-protein nitrogen	Urea and ammonia nitrogen	Total non-protein nitrogen	Urea and ammonia N	
Before....	88	23	78	20	<i>per cent</i> 26
After.....	50	11	48	11	23

It is seen that in none of the experiments with glycocoll is there any suggestion of urea formation (not even in Experiments 10 and 11, in which a considerable amount of nitrogen was washed out of the liver), although precisely similar experiments with ammonium carbonate demonstrate the ability of the surviving liver under these conditions to metabolize considerable amounts of ammonia, and, in the two experiments in which data are available (4 and 6), to form urea, or some nitrogenous substance not blown over by the air-current, but hydrolyzed at 150°C. under the conditions of the determination. The concentrations of glycocoll added have been about those used by Salaskin.

In the introductory part of this paper we have offered a number of possible explanations of this difference in results. It is not at all likely that the difference in the animals used would lead to so different results.

We by no means wish to argue, from the results obtained, that urea formation from amino-acids does not occur in the liver at all, for such a conclusion would be quite unjustifiable on the basis of the above data. We do believe, however, that it is extremely doubtful that such a process has ever been demonstrated.

The only amino-acid used by us has been glycocoll, but inasmuch as there is no evidence for urea formation from other amino-acids in the liver that is not subject to the same criticism as in the case of glycocoll, we feel justified in making the statement more general.

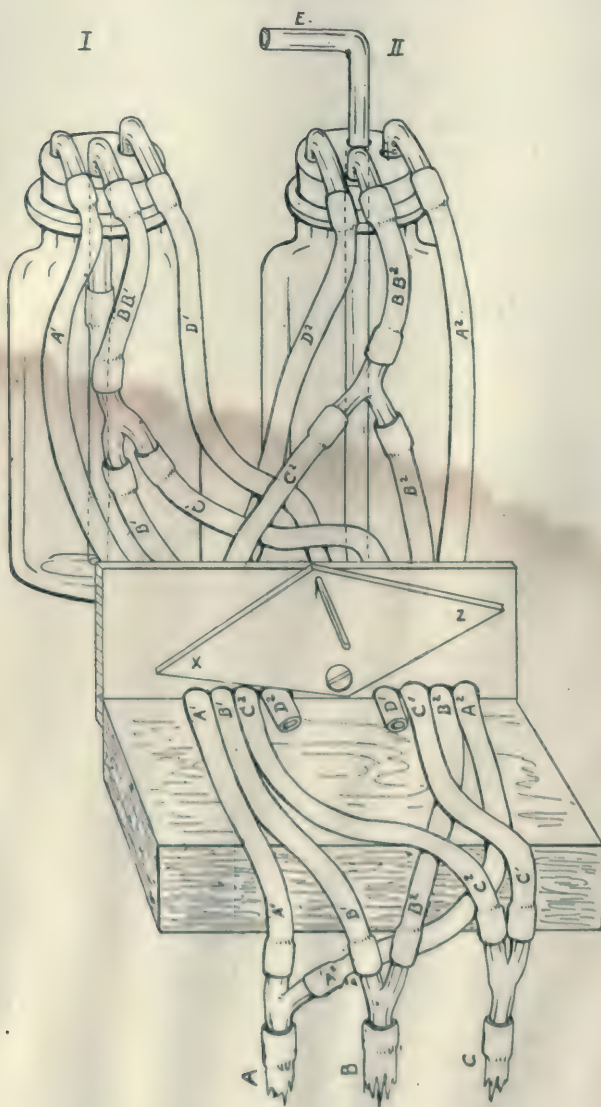
SUMMARY AND CONCLUSIONS.

1. The surviving liver is capable of destroying ammonia perfused through it in the form of ammonium carbonate, and of converting it partially into urea. The entire amount of ammonia changed, however, does not have this fate. How much, if any, of it undergoes synthesis to amino-acids has not been determined. It is doubtful whether the binding of ammonia as such by the liver cells is of much significance in the protective influence of the organ, as indicated by the lack of variation in total non-protein nitrogen content of the fluid during the experiment.

2. The perfusion of the liver of the cat or the rabbit with homologous defibrinated blood containing as much as 44 mgm. of nitrogen as glycocoll per 100 cc. does not lead to any increase in the amount of urea in the fluid used.

3. The formation of urea from amino-acids by the liver is not conclusively demonstrated. There is no incontestable ground for the assumption that the liver is a special site for such a process.

We are greatly indebted to Professor Otto Folin for valuable advice received at various times during the course of this work.



EXPLANATION OF DIAGRAM OF PERFUSION BOTTLES.

Tube *A* is for inlet of air pressure, passing through A^1 or A^2 to the bottles. Fluid passes through B^1 or B^2 to *B*. Fluid returns through *C* to C^1 or C^2 , and thence to bottles. Air is released from bottles through tubes D^1 or D^2 . When the rocker valve is closed on *Z* side, the air pressure reaches bottle *I*, tube D^1 being closed on *Z* side of throttle; fluid passes through tube BB^1 to B^1 , the arm of the Y-tube connecting with C^1 not permitting passage of fluid because of closure by *Z* side of rocker. Fluid passes thence through *B*, through water coil, liver and returns to bottle *II* by way of *C* and C^2 , the latter being open on *X* side of throttle; fluid passes into bottle *II* because air is allowed to escape through D^2 .

D^1 and D^2 were frequently connected with a Y-tube and the exhaust air passes through $\frac{N}{10}$ HCl, but not enough ammonia was detected with Nessler's reagent to affect the figures.

Tube *E* was kept closed by means of a clamp, but when bottle *II* was filling, air freed from ammonia was forced into the bottle so as to aerate the fluid.

With reversal of the rocker, the process was reversed but without noticeable interruption to the outflow through *B* and inflow through *C*, although extremely careful attention had to be given water coil and thermometer at this time.



THE SATURATED FATTY ACID OF KEPHALIN.

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(Received for publication, October 22, 1913.)

The work on the composition of kephalin has made little progress from 1909 until the last few months. Last winter we had in our possession a considerable quantity of the ether-soluble fraction of brain lipoids and hence employed it for preparation of kephalin. The preparation was carried out following the process employed by previous workers.¹ The purification was accomplished by repeatedly dissolving the crude substance in petroleic ether (boiling point, 40–50°C.) and precipitating it with alcohol. This operation was repeated about ten times, until the final product had on drying a light straw-yellow color. The ethereal solution was allowed to flow into the alcohol in a very slow stream, mechanical stirring being employed during the precipitation and at least about one-half hour after the completion of the precipitation. The product was then suspended in acetone, which was allowed to act on it under constant stirring. The final product was a dry, light powder.

At the close of last season the work was completed only so far as it concerned the saturated fatty acids obtainable on hydrolysis of kephalin. There existed conflicting statements regarding their nature, Cousin² having isolated only stearic acid, while Fränkel and Neubauer claimed the presence in kephalin of both stearic and palmitic acids.

Four samples of kephalin were analyzed and in all of them was found on hydrolysis only one saturated fatty acid, namely, stearic

¹ Falk: *Biochem. Zeitschr.*, xiii, p. 153, 1908; xvi, p. 187, 1909; Fränkel and Neubauer: *ibid.*, xxi, p. 321, 1909; Fränkel and Dimitz: *ibid.*, xxi, p. 327, 1909; Parnas: *ibid.*, xxii, p. 411, 1909.

² Cousin: *Jour. d. pharm. et d. chim.*, xxiv, p. 101, 1906; xxv, p. 177, 1907.

acid. The publication of this result was postponed as it was planned to resume the further study of the composition of kephalin in course of the present season. However, there has appeared in course of the summer a very important work by Parnas and his co-workers³ on the nature of the nitrogenous component of kephalin, and in the last number of the *Biochemische Zeitschrift*⁴ another publication, also by Parnas,⁵ on the saturated fatty acids of kephalin, in which the writer arrives at the conclusion that stearic acid is the only saturated fatty acid present in the molecule of kephalin.

We therefore concluded to present the results of our last year's work not only because they strengthen the conclusions of Parnas, but also for the reason that in four out of five experiments the separation of the saturated from unsaturated fatty acids was carried out by a process different from the one employed by previous workers.

The separation was based on the difference in the solubility of the ethyl esters of the saturated and unsaturated acids. At 0°C. the saturated acid precipitates out of the alcoholic solution, while the unsaturated still remains in solution. The separation of the saturated acid is perhaps not absolutely quantitative, but very neat and convenient. The results obtained on the acid hydrolysis of kephalin were corroborated by the result obtained on alkaline hydrolysis by means of a barium hydrate solution.

EXPERIMENTAL PART.

The kephalin used in this investigation was purified by repeatedly dissolving it in 40–50° petroleum ether and pouring the solution with stirring into alcohol. Two different samples were analyzed for nitrogen and phosphorus.

³ Baumann: *Biochem. Zeitschr.*, liv, p. 30, 1913; Renall: *ibid.*, lv, p. 296, 1913.

⁴ Parnas: *Ibid.*, lvi, p. 17, 1913.

⁵ Parnas, referring to the article by one of us (Levene) in this *Journal*, xv, p. 153, attributes to the writer the statement that the fatty acid was obtained on hydrolysis of a lipid by means of alcohol acidulated with sulphuric acid. This was an error on the part of Parnas, for the reason that it is clearly stated in the article referred to that the ethyl ester was the first product obtained on the hydrolysis of the lipid with acidulated alcohol.

- I. 0.2988 gram substance gave 0.0430 gram $\text{Mg}_2\text{P}_2\text{O}_7$.
 0.2004 gram substance required 2.15 cc. $\frac{\text{N}}{10}$ HCl (Kjeldahl).
 II. 0.3058 gram substance gave 0.0440 gram $\text{Mg}_2\text{P}_2\text{O}_7$.
 0.4152 gram substance required 4.8 cc. $\frac{\text{N}}{10}$ HCl (Kjeldahl).

	Found:	
	I	II
P.....	4.01	4.01
N.....	1.50	1.62

Hydrolysis with alcoholic HCl.

Fifty grams of kephalin were heated with 600 cc. methyl alcohol and 20 cc. concentrated sulphuric acid ten hours under a reflux and in an atmosphere of carbon dioxide. At the end of the heating there was a slight mineral residue. The colored solution was decanted from this residue and cooled in the ice box over night. The precipitate which formed was filtered off and recrystallized from acetone. It melted at 37–38°. Methyl stearate melts at 38°. The ester was hydrolyzed by heating with alcoholic potassium hydroxide, the soaps decomposed by warm hydrochloric acid, washed free from acid and recrystallized from acetone. The first fraction was analyzed (I), then further purified by changing into the lead salt and decomposing with hydrogen sulphide in toluene; this was then recrystallized from acetone and the last trace of solvent removed by melting. The mother liquor from (I) was concentrated and the acid which separated analyzed (II). Four different hydrolyses were carried out with similar results. The four samples showed m.p. of 69°, 69°, 68–69°, 69°, and gave no depression when mixed with a sample of Kahlbaum's stearic acid, purified through the lead salt.

Analysis of the acid.

- I. 0.1280 gram of the substance gave 0.3557 gram CO_2 and 0.1492 gram H_2O .
 II. 0.1388 gram of the substance gave 0.3860 gram CO_2 and 0.1576 gram H_2O .

	Calculated for $\text{C}_{18}\text{H}_{35}\text{O}_2$	Found:	
		I	II
C.....	76.00	75.79	75.84
N.....	12.70	13.05	12.71

Molecular weight estimations.

Samples from four different hydrolyses were used, all of which were purified through the lead salt.

I. 1 gram of the acid dissolved in a mixture of absolute methyl alcohol and benzene, when titrated with $\frac{N}{2}$ alkali, using phenolphthalein as an indicator, required 34.8 cc. $\frac{N}{10}$ NaOH for neutralization.

II. 1 gram of the acid, as above, required 34.9 cc. $\frac{N}{10}$ NaOH.

III. 1 gram of the acid, as above, required 34.9 cc. $\frac{N}{10}$ NaOH.

IV. 1 gram of the acid, as above, required 34.7 cc. $\frac{N}{10}$ NaOH.

M. W.	Calculated for		Found:			
	$C_{18}H_{36}O_2$:	I	II	III	IV	
	286	287	286	286	285	

Aqueous barium hydrolysis.

A fifth sample of the same kephalin was hydrolyzed by boiling with aqueous barium hydrate in an autoclave for several hours. The mixture of barium salts was filtered off, the unsaturated acid removed by repeated extraction with ether, the saturated acid liberated with warm dilute hydrochloric acid, changed into the lead salt, decomposed with hydrogen sulphide and recrystallized from acetone. The acid thus obtained melted at 68–69°. Mixed with pure stearic acid, it melted at 68–69°. No trace of any other saturated fatty acid could be found.

1 gram of the acid, as above, required 34.6 cc. $\frac{N}{10}$ NaOH for neutralization.

M. W.	Calculated for		Found:
	$C_{18}H_{36}O_2$:		
	286		288

THE INFLUENCE OF BUTTER-FAT ON GROWTH.¹

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With the Coöperation of EDNA L. FERRY and ALFRED J. WAKEMAN.

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(Received for publication, November 4, 1913.)

We have recently pointed out² that while young rats grow for a time at a normal rate on the "protein-free milk" diet used in our earlier experiments, they sooner or later cease to grow, so that they rarely attain more than two-thirds of the weight normal for fully grown rats receiving a diet chiefly composed of milk and lard. Furthermore, we showed that rats which had ceased to grow and were declining on a "protein-free milk" diet, at once recovered and resumed a normal rate of growth when a part of the lard in their food was replaced by a quantity of unsalted butter corresponding to that in the milk-food. The striking way in which butter, thus supplied, influenced the growth of these young rats made it evident that it furnishes some substance which exerts a marked influence on growth.

These observations have since been verified by numerous additional experiments and an attempt has been made to determine with which of the components of the butter this growth-promoting power is associated. As is well known, butter consists of about 82-83 per cent of the glycerides of numerous fatty acids, about 15 per cent of water containing each of the soluble constituents of milk, and from 1 to 2 per cent of solid matter, consisting chiefly of cellular débris from the mammary glands, bacteria, calcium

¹ The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

² Osborne and Mendel: The Relation of Growth to the Chemical Constituents of the Diet, this *Journal*, xv, pp. 311-326, 1913.

phosphate, particles of casein, and accidental impurities introduced during the process of making the butter.

In view of the possibility that even an extremely minute quantity of some substance might exert the favorable influence on growth observed in all of our experiments, we separated the butter into three parts, namely the fatty substances, the insoluble solid elements, and the aqueous solution containing lactose, soluble inorganic salts and other soluble components of the milk; and by feeding trials found that the growth-promoting factor was contained in the fat fraction. Further consideration of the two other fractions is therefore unnecessary.

The butter-fat used in the analytical, as well as the feeding experiments here described, was prepared as follows. The butter was melted by heating in a flask immersed in a bath of water not exceeding 45°, and centrifugated for about an hour at a high speed. The melted butter was thus separated into a layer of perfectly clear fat, an opalescent aqueous layer, and a deposit of white solid matter. The clear fat was sucked off with a syringe, and thus separated from all of the other parts of the butter. By this method the use of all solvents was avoided, and any substances which might have been dissolved thereby from the other parts of the butter were excluded.

So much has been written about the significance of phosphatides (lecithin, etc.) in various biological phenomena, and in growth among others, that a careful analysis of large quantities of the butter-fat was instituted to detect the presence of members of this group of so-called lipoids. The butter-fat prepared as described was found to be entirely free from nitrogen and phosphorus and was devoid of any ash-yielding, or water-soluble, components. The absence of phosphatides from the product corresponds with the recent statement of Njegovan³ who concludes that milk contains no lecithin whatever, and suggests that the contradictory claims of other investigators are attributable to inadequate methods of analysis.

Butter-fat, thus prepared, has proved to be quite as effective as butter or milk in promoting the recovery and renewed growth of animals which have ceased, or failed, to grow on the natural

³ Njegovan: *Biochem. Zeitschr.*, liv, p. 78, 1913.

"protein-free milk" dietaries in which lard furnished the fat component. Our previously published experiments⁴ in which similar recoveries were made when *butter* replaced a part of the lard of diets containing the "artificial protein-free milk" are complicated by the fact that butter contains about 15 per cent of buttermilk, and hence the improvement shown by such experiments might be attributed to some constituent of the buttermilk. Experiments with "artificial protein-free milk" and butter-fat are in progress and it is hoped by these to learn whether or not accessory substances other than those contained in the butter-fat are necessary for growth. These experiments are not yet completed.

Charts showing the body-weights of growing rats that had begun to decline on our "protein-free milk" food mixtures, and were supplied with foods having butter or butter-fat introduced,⁵ are appended (see Charts I, II, and III). The composition of the mixtures was as follows:⁶

	Butter foods per cent	Butter-fat foods per cent
Protein.....	18	18
Starch.....	26	26
Protein-free milk.....	28	28
Lard.....	10	10
Butter-fat.....	15.3	18
Buttermilk, etc.....	2.7	0

} butter

The efficiency of the butter-fat, or some component thereof, in specifically promoting growth is further shown in another way. As has already been pointed out, when very young rats are placed on a mixture of purified protein, lard, starch, and "protein-free milk," prepared in imitation of the gross composition of the highly successful milk-food, they show a varying capacity to grow. In some cases growth has stopped after sixty days; other animals have continued to grow for one hundred days or more. But in the growth experiments there has always been an inevitable ulti-

⁴ This *Journal*, xv, p. 326, 1913.

⁵ For similar recoveries induced by milk-food, see Charts II and III, this *Journal*, xv, pp. 321-322, 1913.

⁶ For details regarding the milk-foods used in our experiments, and other comparisons, see Osborne and Mendel: this *Journal*, xv, p. 318, 1913.

mate inhibition of growth, and nutritive decline, connected with some diet factor. Very few of our rats grew after they were 140 days old, at which age two-thirds of the normal growth of the male, and three-fourths of that of the female is usually made. We have accordingly attempted to learn whether the continued exhibition of butter-fat from very early in the growth period would enable the animals to attain their normal maximum weight and thereby avert the invariable failure which hitherto was met with sooner or later. If it be assumed that in these earlier experiences the substance, or substances, essential for growth, and supplied inadequately, or not at all, in the artificially prepared diets, is furnished by a reserve stored in the cells of the young animals, this must be exhausted after a time, and lead to failure of growth. If, however, a growth-promoting substance is present in the butter-fat, and the latter is supplied in abundance, growth ought to continue to its logical conclusion, in the absence of other inhibitory factors.

Experiments to test this were begun with young rats whose diet from an early period consisted of food of the following composition:

	per cent
Purified protein.....	18
Starch.....	26
Protein-free milk.....	28
Lard.....	10
Butter-fat.....	18

In harmony with the theory outlined above, the outcome of these newer feeding trials already demonstrates a far more successful continuance of growth on the foods containing butter-fat than on any other artificial diet-mixture (except milk-food) hitherto tested. An illustrative chart (IV) is appended.

In further corroboration of the efficiency of the butter-fat in promoting growth, and particularly after a previous decline, we may cite additional experiments involving the use of centrifugated, or so-called skimmed milk as the basis of the ration. A food paste, intended to resemble our successful milk-food, was prepared by the use of dried centrifugated milk.⁷ The product used

⁷Like the whole milk this was supplied to us in powdered form by the Merrell-Soule Company of Syracuse, N. Y.

contains only 1.18 per cent of fats. The food had the following composition:

	per cent
Dried centrifugated milk.....	44
Starch.....	28
Lard.....	28

This food, although not entirely free from milk-fat, contained only 0.52 per cent, *i.e.*, less than one twenty-fifth as much as the whole milk-food used for our earlier experiments. Although rats have grown normally upon our whole milk-food for more than a year, the centrifugated milk-food has in every case failed to prove adequate for a comparable period. It is true that the centrifugated milk-food has been more satisfactory as a growth ration than our "protein-free milk" foods, *i.e.*, the moment of ultimate failure to grow or to decline has been postponed longer. This may well be due to the small quantities of the essential butter-fat still present in the commercial product used by us. However, the substitution of a part of the lard in the food-pastes by an amount of butter equivalent to that naturally present in our milk-foods brought prompt recovery, as exemplified in the appended chart V.

The outcome of these experiments clearly indicates that the growth-promoting substance of the milk is to be found in the butter-fat fraction thereof; for the two rations here illustrated and characterized by either nutritive failure or success, differ in respect to the fat component only. The influence of heating and other processes involved in the preparation of milk for food were also incidentally investigated. These studies, though far from completed, have given no evidence, in so far as nutrient efficiency is concerned, of a damage to the centrifugated milk by vigorous sterilization. We hope to return to this question in a later communication.

The experiments recently reported by McCollum and Davis,⁸ who added an ether extract of butter or eggs to artificially prepared food-mixtures, are in accord with the results which we have obtained by feeding butter, or butter-fat, to rats which were declining after a period of growth on a diet of isolated food-sub-

⁸ Cf. McCollum and Davis: The Necessity of Certain Lipins in the Diet during Growth, this *Journal*, xv, p. 167, 1913.

stances. In none of their published records was the recovery rapid as in most of ours, nor was the rate, or extent, of growth after reaching the previous maximum weight, any greater than on the butter-fat-free diet earlier supplied. In the experiments they report, the gain made by their rats after recovering to their previous maximum weight was 40, 25, 40, and 60 grams, a part of the latter gain being made after the rat had eaten her own young. The time during which the rats were fed on diets containing the ether extract was 5, 9, 12, and 12 weeks, but in no case did the animal reach a weight normal for its age. Although the data furnished by McCollum and Davis strongly indicate that butter-fat has a marked influence on growth they by no means prove that butter-fat contains something essential for the metabolism of growth, apart from that of maintenance. The added butter-fat may have simply supplied something analogous to the so-called vitamins, which Funk considers to be essential for life, and thereby enabled the animals to resume growth on a food thus made adequate for maintenance. Until it is shown that rats can be maintained for long periods on such diets as McCollum and Davis used no final conclusion can be drawn respecting the above question.

By numerous experiments we have shown that mature rats can be maintained on our "protein-free milk" diets for more than a year, and that young rats on similar diets containing proteins inadequate for growth can be maintained nearly as long. Such foods consequently supply all that is essential for *maintenance* alone. Since growth ceases on these foods after a comparatively short time, and is at once resumed and continued throughout the entire period of normal growth when a part of the lard is replaced by butter-fat, it is almost certain that butter-fat contains something essential for growth in addition to what may be required for maintenance. This recovery and renewed growth must be attributed to something which distinguishes butter from the ordinary fats, for not only do lard and olive oil⁹ lack this growth-promoting power, but young rats grow on our "protein-free milk" foods when all of the lard is replaced by carbohydrate¹⁰ and no ether-soluble substances are present in the food.

⁹ McCollum and Davis: *loc. cit.*

¹⁰ Cf. Osborne and Mendel: Feeding Experiments with Fat-free Food Mixtures, *this Journal*, xii, pp. 81-89, 1912.

It thus appears improbable that glycerides of the fatty acids ordinarily present in foods are responsible for the promotion of the growth observed when butter-fat replaces lard in the diet of rats which have ceased to grow. Lecithin and other phosphorus- or nitrogen-containing substances are excluded by the absence of phosphorus and nitrogen from our butter-fat; and cholesterol by the fact that even more of this substance has been obtained from lard than from butter.¹¹

So far as our experience has shown, the addition of butter-fat to our natural "protein-free milk" foods gives them an efficiency quite comparable with that of our milk-food in promoting recovery and the completion of growth. The exact chemical differences between the adequate butter-fat and the inadequate lard (which determine success and failure respectively in the food-mixtures employed) are far from being satisfactorily known. Chemical examination of the butter-fat indicates that the effective component is not a phosphatide or any inorganic substance, inasmuch as nitrogen, phosphorus and ash are lacking in the product employed. It is suggestive to note that in the one case (lard) we are dealing essentially with a fat-mixture deposited in storage depots of the animal organism; in the other, the butter-fat represents the product of metabolic activity and synthesis on the part of the cells of the mammary gland. What, if anything, this distinction between cellular product and reserve fat may mean physiologically, remains to be investigated.

The researches which have been devoted in recent years to certain diseases, notably beri-beri, have made it more than probable that there are conditions of nutrition during which certain essential, but, as yet, unknown substances must be supplied in the diet if nutritive disaster is to be avoided. These substances apparently do not belong to the category of the ordinary nutrients, and do not fulfil their physiological mission because of the energy which they supply. Funk has proposed the name *vitamine* for the type of substance thus represented.¹²

Without minimizing the importance of the new field of research and the new viewpoints in nutrition which are presented by these

¹¹ Cf. McCollum and Davis: *loc. cit.*, p. 174, who fed cholesterol to rats.

¹² The literature on the subject has been reviewed by Funk: Ueber die physiologische Bedeutung gewisser bisher unbekannter Nahrungsbestandteile der Vitamine, *Ergeb. d. Physiol.*, xiii, p. 125, 1913.

recent findings, we may nevertheless hesitate to accept the extreme generalizations which have already been proposed on the basis of the evidence obtained largely from the investigation of pathological conditions. The statement, for example, that a "tadellose Nahrung" may prove entirely inadequate unless "vitamines" are present, at once suggests a series of questions bearing on what is included in the new term. It is still rather early to generalize on the rôle of accessory "vitamines" when the ideal conditions in respect to the familiar fundamental nutrients and inorganic salts adequate for prolonged maintenance are not completely solved. Speculation is quite justifiable in so far as it directs attention to a new phase that needs to be taken into account.

Funk has expressed the belief that the substance which promotes growth and must be present in order to avert the cessation of growth, which we have described to occur after a certain period of successful growth on our earlier dietaries, is either identical with, or analogous to, the "vitamine" which plays the rôle of an antiscorbutic substance. For this we can as yet find no compelling evidence. Certainly the nitrogen-free butter-fat, so successful in remedying our growth failures, contains no substance chemically related to the nitrogenous products which have lately been credited with this unique physiological efficiency.¹³ Furthermore it

¹³ Cf. Funk: Ueber die physiologische Bedeutung gewisser bisher unbekannter Nahrungsbestandteile der Vitamine, *Ergeb. d. Physiol.*, xiii, p. 130 *et seq.*, 1913. In reviewing our earlier published experiments Funk has erroneously assumed that we secured *completed* growth with the diets in which the butter component was not yet employed. It is true that the increments in weight were in some cases very noteworthy; but in every instance cessation ultimately ensued before the completion of the normal progress of the growth or subsequent maintenance. We have never denied the necessity of a growth-promoting food accessory in accord with the claim of Hopkins; and recently we pointed out that the successful partial completion of growth, such as has been obtained in our experiments, may well have been due to a store of the essential compound in the body of the experimental animals at the beginning of the trials. It is by no means necessary to assume with Funk that small quantities of these accessory substances were inadvertently left in our food preparations owing to insufficient extraction with alcohol.

Furthermore we cannot agree with Funk that the rat is not well adapted to experiments on the physiology of growth. The superiority of this animal has been pointed out by us elsewhere (cf. this *Journal*, xiii, p. 233,

is well to bear in mind that it is not improbable that the anti-neuritic and antiscorbutic constituents of foods are not identical with the substances alleged to assist in maintaining body-weight.¹⁴ Funk¹⁵ has lately asserted that the simultaneous administration of at least two substances is necessary to produce the curative effect obtained in his previous experiments with the "vitamine" fraction from rice-bran or yeast. Voegtlin and Towles¹⁶ have noted that extracts of autolyzed spinal cord may be antineuritic, yet be unable to reestablish normal metabolism, *i.e.*, restore body-weight.

Butter-fat has shown a further interesting nutritive superiority over lard. At certain periods of the year, particularly in summer months, we have frequently failed to secure satisfactory growth on the dietaries which proved adequate during the usual period of sixty to one hundred days at other seasons. Occasionally young rats in the stock colony have exhibited a similar "epidemic" of poor growth at the same season. The failures are, however, not common to rats fed on the milk-food; and we have lately observed that the seasonal failure is also averted by the addition of butter-fat to the usual "protein-free milk" food-mixtures.¹⁷ Again, another type of nutritive deficiency exemplified in a form of infectious eye disease prevalent in animals inappropriately fed¹⁸ is speedily alleviated by the introduction of butter-fat into the experimental rations.

The chemical character of the unique "accessory substance" in butter-fat must be investigated in detail and its possible pres-

1912) and is also apparently recognized by both Donaldson and McCollum and their coworkers. We have found rats to be responsive to changes in diet; and we count it no disadvantage that the experiments must be continued over sufficient time to exclude minor incidental fluctuations.

¹⁴ Cf. Cooper: *Journ. of Hygiene*, xii, p. 433, 1912.

¹⁵ Funk: *Ergeb. d. Physiol.*, xiii, p. 547, 1913; *Brit. Med. Journ.*, April 19, 1913; *Journ. of Physiol.*, xvi, p. 173, 1913.

¹⁶ Voegtlin and Towles: *Journ. of Exp. Pharmacol.*, v, p. 67, 1913.

¹⁷ These summer failures in growth have been reported to us by colleagues to occur likewise in other institutes.

¹⁸ Cf. Knapp: Experimenteller Beitrag zur Ernährung von Ratten mit künstlicher Nahrung und zum Zusammenhang von Ernährungsstörungen mit Erkrankungen der Conjunctiva, *Zeitschr. f. exp. Path.*, v, pp. 147-170, 1908

ence elsewhere determined. Experiments are already under way with varying proportions of butter-fat in the ration; but we have not thus far determined the necessary allowance. On the other hand, no amount of butter-fat will induce growth on certain diets in which the proportions and nature of the inorganic salts are inappropriate (as in our Salt mixture I),¹⁹ or the quantity and character of the protein is inadequate. The "Bausteine" must not be overlooked in our enthusiasm for these newer features.

ADDENDUM. An investigation now under way to determine the possible efficiency of fats other than butter-fat in preventing decline on our protein-free milk-food and promoting growth in the way that butter does, has already indicated marked differences in fats from different sources. Egg yolk-fat, for example, appears to behave like butter-fat; some other oils have thus far proved no more efficient than lard. Such considerations make it evident that the comparative value of the natural fats employed in nutrition must be determined, as well as the individual rôle of the different proteins, carbohydrates, and mineral nutrients.

¹⁹ See Osborne and Mendel: Carnegie Institution of Washington, Publication 156, pt. ii, p. 80.

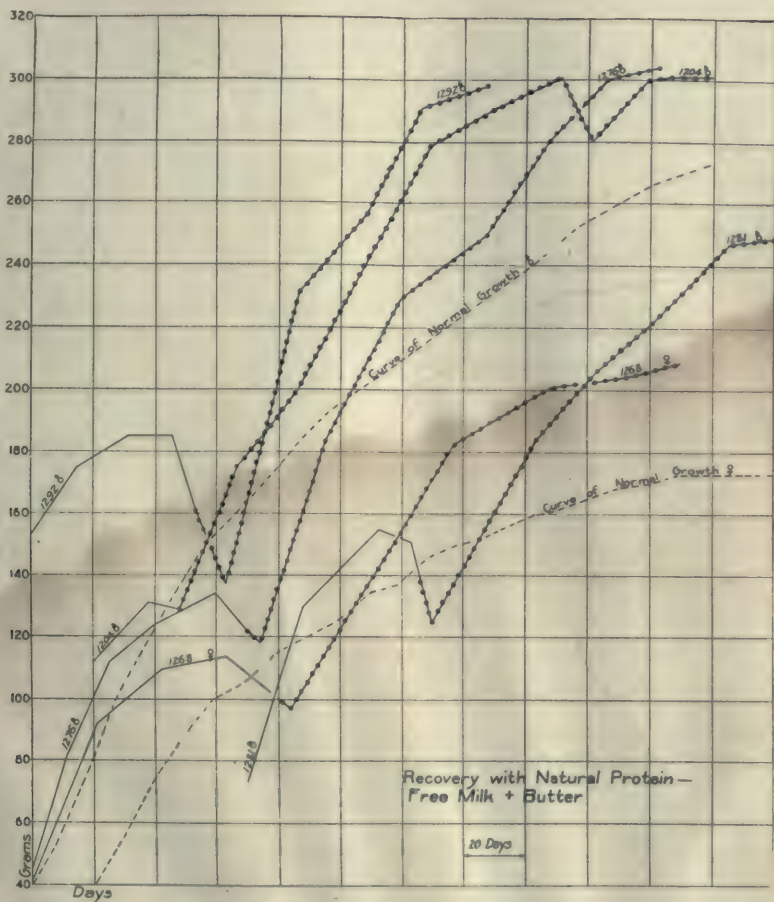


CHART I. Curves of body-weight of rats which have ceased to grow and have declined on foods containing the natural "protein-free milk," and have recovered when 18 per cent of unsalted *butter* replaced the same quantity of lard in the diet, as indicated by the interrupted lines (-o-o-o-o-o). The proteins furnished in the different experiments were as follows: casein, Rats 1204, 1268, 1276, 1281, 1292; ovalbumin, Rats 1268, 1276.

The ordinates represent grams of body-weight, as indicated. The divisions of the abscissa represent 20-day periods.

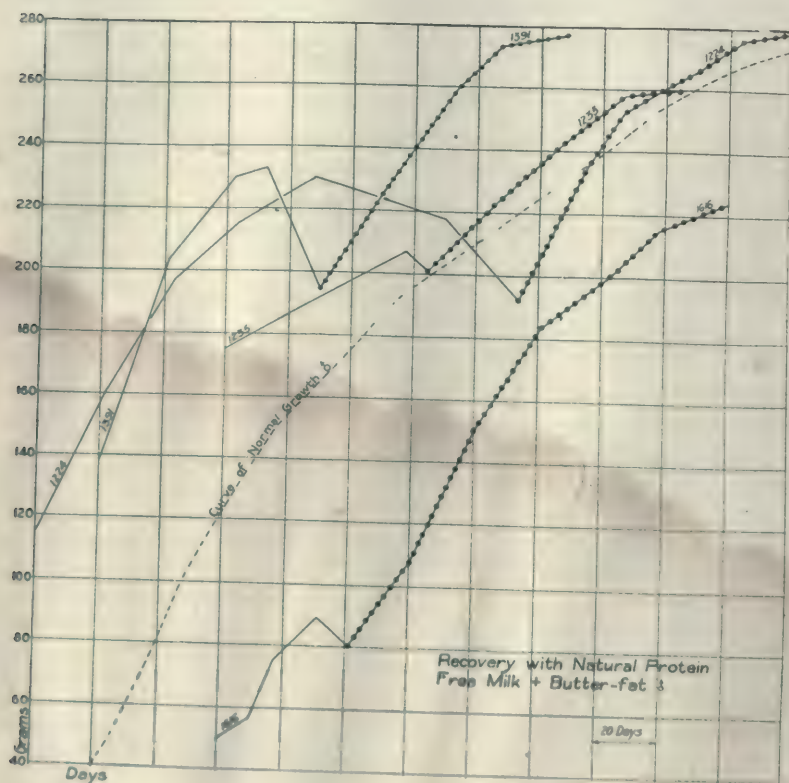


CHART II. Curves of body-weight of male rats which have ceased to grow and have declined on foods containing the natural "protein-free milk," and have recovered when 18 per cent of *butter-fat* replaced the same quantity of lard in the diet, as indicated by the interrupted lines (-o-o-o-o-). The proteins furnished in the different experiments were as follows: casein, Rats 1224, 1235; edestin, Rat 1391; zein + casein, Rat 1616.

The ordinates represent grams of body-weight, as indicated. The divisions of the abscissa represent 20-day periods.

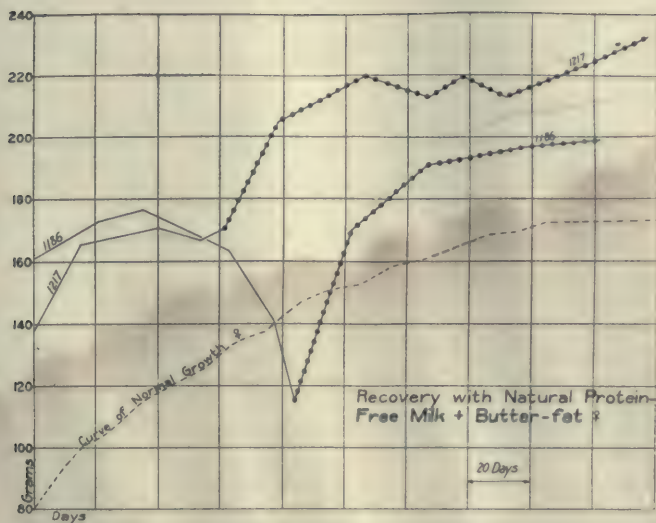


CHART III. Curves of body-weight of female rats which have ceased to grow and have declined on foods containing the natural "protein-free milk," and have recovered when 18 per cent of *butter-fat* replaced the same quantity of lard in the diet, as indicated by the interrupted lines (-o-o-o-o-). The proteins furnished in the different experiments were as follows: casein, Rat 1217; zein + lactalbumin, 1186.

The ordinates represent grains of body-weight, as indicated. The divisions of the abscissa represent 20-day periods.

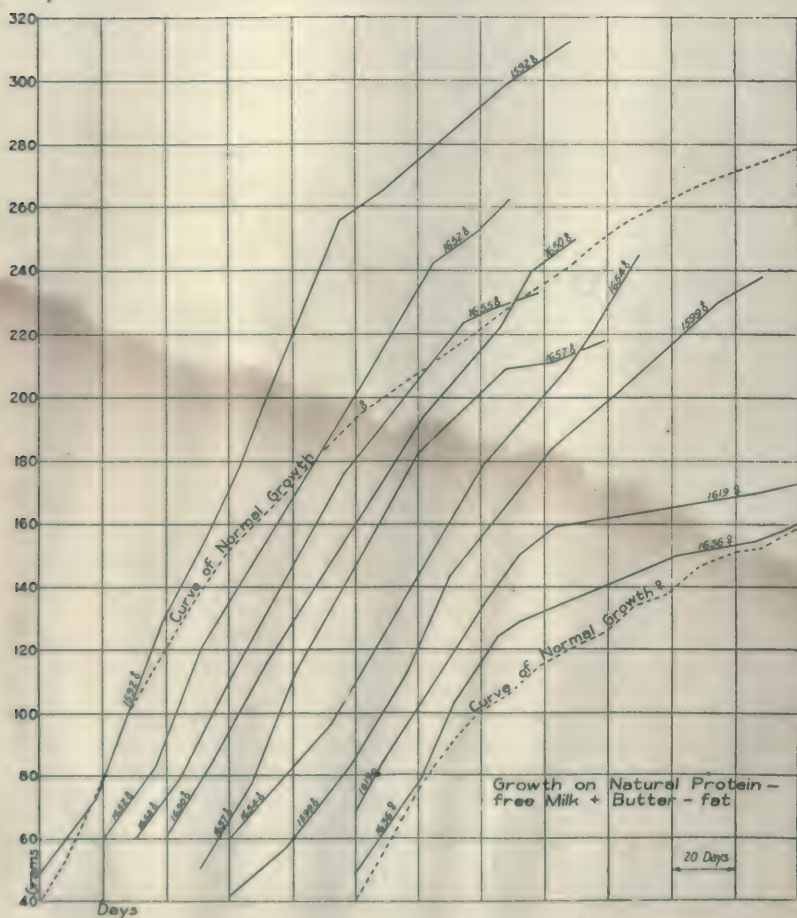


CHART IV. Typical curves showing prolonged normal growth of white rats on foods containing 18 per cent of butter-fat. The proteins furnished in the different experiments were as follows: casein, Rats 1592, 1599, 1619, 1636, 1652, 1655, 1657; edestin, Rats 1650, 1654.

The ordinates represent grams of body-weight, as indicated. The divisions of the abscissa represent 20-day periods.

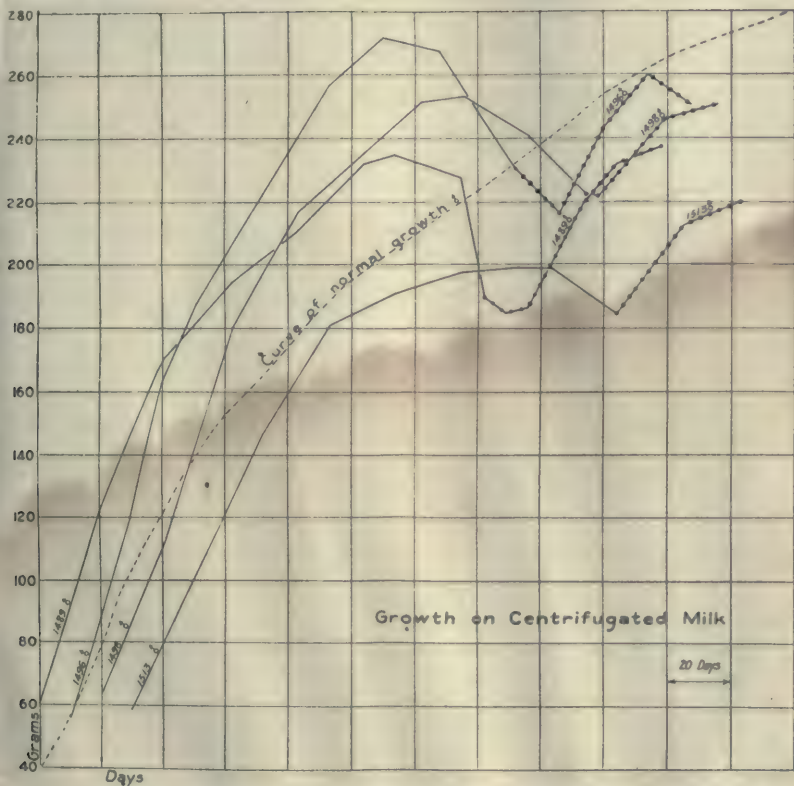


CHART V. Curves of the body-weight of rats which have ceased to grow and have declined on the *centrifugated-milk-food*, and have recovered when 18 per cent of *butter-fat* replaced the same quantity of lard in the diet.

The ordinates represent grams of body-weight, as indicated. The divisions of the abscissa represent 20-day periods.



THE EFFECT OF FERMENTS AND OTHER SUBSTANCES ON THE GROWTH OF BURLEY TOBACCO.

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The growth of the tobacco plant during the first stage is very slow compared with some of the plants comprising the general farm crops. This is shown by the fact that it requires about two months or more in the seed bed to become of sufficient size for transplanting. In other words, it takes the young plant from the time the seed is sown until it reaches this stage, from one-half to three-fifths as long as it requires after transplanting to grow to maturity. Therefore, it can readily be understood that if some way could be found of shortening the first period of growth until the transplanting stage is reached, this would save the grower considerable time in looking after the seed beds as well as decreasing the chances of insects, fungi and bad weather from injuring and destroying the plants. Furthermore, in sections of the country where tobacco might be grown but for the short season, this would be a benefit in assisting to produce a crop. The grower realizes these difficulties and takes advantage of all the best conditions for obtaining the largest plants in the seed bed in the shortest time by locating it in virgin soil with the best exposure and applies fertilizers if necessary.

A great deal of work has been done in the past few years on the changes taking place in the germination and growth of seeds. It is now generally recognized that these changes depend primarily upon what are commonly called ferments or enzymes. The seed, as is well known, contains a certain amount of reserve food material which nourishes the seedling and enables it to grow until it can draw on the soil for its future supply. This stock of food in the seed may be in the form of carbohydrates, oils, fats, protein, etc., and the functions of the different enzymes are to act

upon their particular part of this food supply and convert it into soluble form which can be utilized by the seedling.

F. A. Waugh¹ has shown that by soaking old seeds of some plants in different enzyme solutions, higher percentages of germination could be obtained than in the same seeds soaked in water. He worked with tomato, cucumber, radish and watermelon seeds of different ages, from five to twelve years old, and obtained increased germination in some which had been soaked in diastase solution, given in the following table:²

Influence of enzymes upon germination.

Description of seeds	Solution employed	Per cent germination
Tomato, 12 years old.....	Water.....	12
“ “ “ “	Diastase.....	85
“ “ “ “	Water.....	34
“ “ “ “	Diastase.....	70
“ “ “ “	Water.....	14
“ “ “ “	Diastase.....	24
“ 5 “ “	Water.....	36
“ “ “ “	Diastase.....	46
Cucumber 5 years old.....	Water.....	44
“ “ “ “	Diastase.....	54
Radish, 6 years old.....	Water.....	46
“ “ “ “	Diastase.....	66

The tomato seeds above were of different varieties and the plan was followed of soaking the seed in water and the ferment solution for the same time, then draining and transferring them to the germination apparatus.

The tentative conclusions reached by Waugh were:

(1) In some cases the percentage of germination in seeds is greatly increased by soaking for several hours in a solution containing some active enzyme or enzymes.

(2) The vigor of the young plantlets is often enhanced at the same time.

(3) Within limits these beneficial effects increase with the strength of the enzyme solution.

(4) Diastase, either from malt or from various commercial preparations, seems to be most useful.

¹ Tenth Annual Report, Vermont Agric. Exp. Station, 1896, p. 106.

² Taken from the Research Bulletin No. 22, p. 105, of the Wisconsin Agricultural Experiment Station.

(5) Tomato seeds seem to respond especially well to the action of enzymes, particularly to the action of diastase.

S. M. Babcock³ has found in a similar experiment that corn, less than 50 per cent of which germinated when the seeds were soaked in water, all germinated when they were soaked in commercial diastase solution. The maximum growth was about the same in each lot, but the growth of the seeds soaked in diastase was very uniform, while that of the water lot varied greatly. The increased vitality of the diastase lot was very noticeable.

He also found that seeds from the same lot that were soaked for fifteen hours in a 3 per cent glucose solution, instead of water, all germinated, thus confirming the view that lack of suitable food was the chief reason why the untreated seed germinated poorly. In this case, there was probably a lack of a starch-inverting enzyme in the seed since equally good results were obtained when either diastase or glucose was supplied.

Among other investigators who have obtained good results working in the same manner may be mentioned A. Thomson,⁴ who obtained excellent results on the seeds of barley, oats, corn, peas, white and yellow clover, using 5 per cent pepsin and diastase solutions separately. Others might still be mentioned who have obtained increased germination and vigor of growth of the seedlings by employing these and solutions of other enzymes, but it is not necessary to mention them here.

From the literature at hand, it was found that very few, if any, experiments have been made by supplying ferments or substance which ferments act upon to the growing plant, but the work seems to have been confined chiefly to the germination of seeds and the initial growth of the young seedling. No reference has been found where any work of this kind has been done on tobacco.

In view of the fact that in some recent work⁵ the writers have found several enzymes in the growing tobacco and also in the seed, it was thought it might prove of interest to try the effect of supplying dilute solutions of some of the ferments, or of the materials which they act upon, to the young plants to see if they

³ Wisconsin Agricultural Experiment Station, Research Bulletin No. 22, p. 106.

⁴ *Gartenflora*, Berlin, xlv, p. 344, July, 1896.

⁵ *Journ. Amer. Chem. Soc.*, xxxv, No 9, September, 1913.

would promote their growth. It was thought that the seed might be lacking either in a part of the necessary food reserve material or that one or more of the enzymes might not have sufficient activity to promote the desired changes required by the growing plant. A brief outline of the plan of the experiments was as follows:

Before sowing, the seeds were soaked in the solutions of the different substances and after germinating in the soil, the young plants were supplied with fresh dilute solutions of the same until they had reached the transplanting stage in the greenhouse. In the meantime, observations were frequently made as to the uniformity, thickness and growth of the plants in the different boxes; and at the end the plants in each were cut close to the soil and weighed so that more definite results could be obtained in regard to what the experiments had demonstrated.

For the work, a sufficient quantity of virgin bluegrass soil was sterilized by heating at 90° – 100°C . for thirty minutes to destroy the weed seed. The soil was then thoroughly mixed and equal quantities of about ten pounds were measured into boxes $12 \times 12 \times 3$ inches in size and supplied with good drainage. Two hundred Burley tobacco seeds that had been cleaned were used for each box and each lot was soaked in the different solutions for twenty-four hours, filtered and allowed to dry over night before sowing. The amount of the different substances used in the above solutions, the manufacturer and the box number in the series were as follows:

No. 1. Burley seed + 2.5 cc. of hydrant water used as a check.

No. 2. Same as No. 1.

No. 3. Burley seed + 2.5 cc. of 5 per cent peptone solution (Witte).

No. 4. Burley seed + 2.5 cc. of 5 per cent diastase of malt solution (Eimer & Amend).

No. 5. Burley seed + 2.5 cc. of 5 per cent Taka-diastase solution (Parke, Davis & Co.).

No. 6. Burley seed + 2.5 cc. of a nutritive solution containing 1500 cc. hydrant water + 1 gram KNO_3 + 0.5 gram $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ + 0.5 gram $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ + 0.5 gram $\text{Ca}_3\text{P}_2\text{O}_8$.

No. 7. Burley seed + 2.5 cc. of 5 per cent glucose solution (Eimer & Amend).

No. 8. Burley seed + 2.5 cc. of 5 per cent trypsin solution (Fairchild Bros. & Foster).

No. 9. Burley seed + 2.5 cc. of 5 per cent pancreatin solution (Merck, pure).

No. 10. Burley seed + 2.5 cc. of 5 per cent papain solution (Eimer & Amend).

11. Burley seed + 2.5 cc. of 5 per cent casein solution (acc. to Hammarsten).

No. 12. Burley seed that had been treated with H_2SO_4 .

No. 13. Burley seed + 2.5 cc. of a mixture of equal parts of 5 per cent Taka-diastrase and 5 per cent pepsin solutions.

No. 14. Burley seed + 2.5 cc. of 5 per cent pepsin solution (Merek, U. S. P., VIII).

No. 15. Burley seed + 2.5 cc. of 5 per cent emulsin solution (Kahlbaum).

No. 16. Burley seed without any previous soaking with water. This was used as a check to compare the unsoaked seed.

No. 6 was carried on so that a comparison might be made of plants that had been supplied with a solution containing all the necessary plant food.

No. 12 was included in order that the plan recommended by Love and Leighty,⁶ of acid treatment of some hard coated seeds⁷ to insure a quicker and better germination, might be tried. This treatment was as follows: A quantity of seeds were soaked with five or six times their volume of H_2SO_4 (sp. gr. 1.84) for fifteen minutes. Water was then added and decanted quickly, so as to prevent the seed from heating. The seeds were then washed with water until free of acid by litmus paper test and dried.

On January 15, the seeds were mixed with sand and sown in their respective boxes and to each were added 800 cc. of water. After this date, the boxes were watered frequently, adding each time 500 cc. of a fresh 0.01 per cent solution of the substances given above to the respective boxes, except No. 13 to which were added 500 cc. of a solution containing 0.005 per cent of each ferment. Of course No. 6 was watered only with the nutritive solution and Nos. 1, 2, 12 and 16 with water. To each box, however, the same volume of 500 cc. was added every time. The nutritive solution was not used constantly but alternated with water. Hydrant water was used throughout the experiments.

From the beginning Nos. 14, 13 and 11 gave the best growth and Nos. 14 and 11 maintained this lead until the plants were weighed. Nos. 10, 15, 9 and 7 also showed up well from the first. Nos. 6 and 3 made good gains in the last few weeks of the

⁶ Bulletin 312, Cornell Agric. Exp. Station, p. 335.

⁷ Tobacco seeds were not used in Love and Leighty's experiments.

experiment. After January 25, the strength of each solution except No. 6, was reduced to one-half of that formerly used because it was thought that this would lessen the chances of fungi developing in the boxes.

The boxes were watered on an average two and one-half to three times a week using the same amount of water or solution during the first part of the experiment. Afterwards it was found that the boxes which were making the better growth appeared to retain the water longer than the others. The plan was then followed of adding extra water to the dry boxes so as to keep all at about the same degree of wetness. Consequently the checks and those boxes which gave the poorest growth had more water added.

It is interesting to note that the boxes which gave the better growth of plants were the ones which seemed to retain the moisture longer and this was true from the beginning. This was very noticeable during the first part of the experiment on comparing these boxes with the checks. It would be expected that as the result of the larger growth of the plants and therefore of their larger water requirement that these boxes would have dried out quicker than those containing the smaller plants, but the reverse seemed to take place. There might be a possible reason for this when the plants attained sufficient size to shade the soil and enable it to hold the water, but even then it would more than likely be counterbalanced by the increased size of the plants. Certainly this explanation would not hold true when all the plants were very small. While the foregoing from frequent observations appeared to be true, further work is required to prove this interesting point.

About the first of March, approximately the same number of plants in each were transplanted and arranged in their respective boxes so that they would be more evenly distributed in the soil.

On March 15, the plants in several of the boxes were of sufficient size to be transplanted. At this time, No. 14 had the largest plants. These appeared to be more uniform in size and thicker in the box. Next in order were Nos. 10, 11, and 15. Then came Nos. 3, 6, 7, 8 and 13 with the plants as evenly distributed and of uniform size as in the boxes mentioned above but somewhat smaller. Next in order was No. 16, and finally Nos. 1, 2, 4 and

5 had about the same appearance. In Nos. 1, 2, 4, 5 and 16 the plants did not come up with an even stand and were as a rule much smaller than the rest. No. 16, however, made a very good growth towards the end and gained on some of the other boxes. This may have been due to the fact that the plants in this box, not being as thick as those in some of the others, had more room in which to grow.

On March 21, the plants were cut off close to the ground and those in each box weighed separately in order to see what differences would be shown in the individual weights. The results were as follows:

No.	Grams	No.	Grams	No.	Gram
1 (check).....	55	6.....	128	11.....	139
2 (check).....	92	7.....	116	12.....	—*
3.....	113	8.....	127	13.....	116
4.....	91	9.....	96	14.....	174
5.....	89	10.....	*126	15.....	138
				16 (check).....	120

* Not weighed; only two seeds germinated.

The above weights of the plants in the different boxes are in fairly close agreement with the conclusions reached as to their general appearance before cutting. The differences between the checks Nos. 1 and 2 are larger than desirable but differences like these will occur in work of this kind and are difficult to explain. The differences between Nos. 1, 2 and 16, all check boxes, are again large and in favor of the unsoaked seed (No. 16), which is contrary to what would be expected. One explanation, although hardly a plausible one, as to why this may be true, is that soaking the seed before planting may have extracted some soluble reserve material from it which the plant needs for its growth. The differences between Nos. 1 and 2, according to this supposition, may have been caused by the water extracting more of this material from one lot of seeds than from the other. The growth of the plants in Nos. 1, 2 and 16 was in harmony with this theory since No. 16 maintained the lead over the others almost from the beginning.

The proper checks on this series are Nos. 1 and 2 and, taking the better or No. 2, the differences between this and some of the best, for example, Nos. 11, 14 and 15 are very large. In only

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three, Nos. 4, 5 and 9 were the results about the same as this check while the remainder show consistent gains over it.

The above results may be due to some fertilizer constituent that was added in the solutions. On the other hand, they may be due to some deficient substance or ferment that was supplied to the plants.

As it is almost impossible to obtain the active ferment free from protein material, it was thought that the total nitrogen in the substances used would probably explain the differences obtained in the growth of the plants. Accordingly, nitrogen determinations were made on all the materials by the modified Kjeldahl method so that any nitrate nitrogen, if present, would be included.

For convenience, the materials used, the weight of the green plants, the increase over check No. 2, the amount of substance supplied and of nitrogen in the same, are given in Table I.

TABLE I.

SUBSTANCE	WEIGHT OF PLANTS WHEN CUT	GAIN IN WEIGHT OVER CHECK NO. 2	WEIGHT OF SUBSTANCE ADDED		NITROGEN IN SUBSTANCE ADDED	TOTAL WEIGHT OF NITROGEN ADDED
	grams	per cent	grams		per cent	gram
No. 1. Check.....	55					
No. 2. Check.....	92					
No. 3. Peptone.....	113	22.8	0.550		15.880	0.0873
No. 4. Malt diastase.....	91	-1.1*	0.550		6.040	.0332
No. 5. Taka-diastase.....	89	-3.3*	0.550		1.400	.0077
No. 6. Nutritive solution...	128	39.2	5.000 of KNO ₃		13.860	.6930
No. 7. Glucose.....	116	26.1	0.550		0.640	.0035
No. 8. Trypsin.....	127	38.0	0.550		12.260	.0674
No. 9. Pancreatin.....	96	4.3	0.550		12.580	.0692
No. 10. Papain.....	126	37.0	0.550		1.560	.0086
No. 11. Casein.....	139	51.1	0.550		14.640	.0805
No. 12. Seed treated with H ₂ SO ₄						
No. 13. { Taka-diastase } { Pepsin }	116	26.1	{ 0.275 0.275		1.400	.0039 5.140 .0141
No. 14. Pepsin.....	174	89.1	0.550		5.140	.0283
No. 15. Emulsin.....	138	50.0	0.550		10.500	.0578
No. 16. Check.....	120					

CONCLUSIONS.

After a period of two months, it appears from the above table that marked differences can be observed in regard to the growth made by some of the plants. For instance, pepsin, casein and emulsin made larger and more uniform plants than the rest. It is interesting to note the good results obtained from the use of emulsin, and in this connection it might be mentioned that good tests were obtained for this enzyme in the Burley seed and growing plant in some previous work.

In every case, with the exception of two, all the boxes were better than the two checks, Nos. 1 and 2, which properly belong to the series and one-half of the boxes were better than the third check, No. 16. As a rule, the proteolytic ferments and the protein substances gave the best growth. The results obtained with trypsin and pepsin are contrary to what would be expected since it is doubtful if the latter is present in plants. Pepsin is regarded as an animal ferment and works best in an acid medium. No positive results were obtained with the diastase ferments.

The results in all cases cannot be explained from the total nitrogen added, since pepsin, which gave the best growth, has only 5.14 per cent of nitrogen, whereas some of the others which did not have so great an effect, as pancreatin, peptone and trypsin, contain respectively 12.58, 15.88 and 12.26 per cent of nitrogen. Again, on comparing pepsin which gave 89.1 per cent increase of growth with malt diastase which showed a decrease of 1.1 per cent, we find that the total nitrogen in the two samples is 5.14 per cent and 6.04 per cent respectively with the advantage in favor of the latter.

To No. 6 was supplied in the nutritive solution a total of 5 grams KNO_3 ; 2.5 grams $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 2.5 grams $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ and 2.5 grams $\text{Ca}_3\text{P}_2\text{O}_8$. This box was certainly supplied with abundant plant food, and while it made a steady growth throughout the experiment, nevertheless pepsin, emulsin and casein made better growths with much smaller amounts of nitrogen supplied. Papain, trypsin and the nutritive solution gave about the same results yet the nitrogen added to each was 0.0086, 0.0674 and 0.6930 gram respectively.

The question of the availability of the nitrogen in the various

substances might explain some of the differences. In this case, the nitrogen supplied in the form of potassium nitrate in the nutritive solution should be readily available. Again, nitrogen in peptone would likely be regarded as more available than in casein yet casein gave a much better growth with less nitrogen added.

Lack of an opportunity prevented any further work from being done on the samples to find if any other fertilizer constituent had been added in appreciable amounts. Granting that some potassium and phosphorus were added in these substances, on the other hand considerably larger amounts of these elements in available form were added in the nutritive solution.

The acid treatment of tobacco seed according to the method used was not satisfactory since only two germinated. The acid was either too strong or the time allowed for soaking the seed too long and this permitted the outer coats to be broken up and partly dissolved, thus allowing the strong acid to come in contact with and kill the embryo.

The substances used in this work were recently obtained from the manufacturer but no previous tests were made on the different ferments to determine their degree of activity.

The plants did not grow as rapidly in the greenhouse as they generally do in the plant bed. When they were cut, the plants in some of the best boxes mentioned above which had reached the transplanting stage were two to three weeks in advance of checks Nos. 1 and 2.

While these experiments are only preliminary, nevertheless some of the results are interesting inasmuch as they indicate benefits that might be acquired in practice. It is intended to continue this work when an opportunity affords and compare these and other materials in which active enzymes are present and the same substances in which they have been destroyed by heat.

PART II.

Many experiments have been made by different investigators on the effect of adding certain substances known as catalytic fertilizers to plants to promote their growth. These substances are generally spoken of under the above title inasmuch as growth

is promoted by their presence and since, as is assumed, they do not necessarily enter into the chemical composition of the plant, they cannot be regarded as plant food materials. They are also referred to as plant stimulants.

Among the substances that have been commonly used are salts of certain metals, such as iron, aluminium, manganese, etc., and while it is only necessary to refer briefly to the literature in regard to the work which has been done along this line, the results of numerous experiments might be mentioned where marked differences in the growth of different plants were obtained on the addition of certain substances to the soil. This is especially true in regard to the use of manganese salts.

The work is in the experimental stage but it opens up an interesting field of investigation in view of the fact that these metals are commonly found in plants and in much larger amounts in the soil. No importance has ever been attached to them other than the fact that one or two are included in the list of the essential elements required by plants, but since they are found in considerable amounts in the soil, it has been assumed that it is not necessary to add them in practice.

Since Euler⁸ mentions the fact that certain substances (for example iron or manganese salts) accelerate the action of some enzymes and as several ferments have been found in the tobacco, it occurred to one of us (Shedd) that it might prove of interest to try the effect of some of these substances on the growth of Burley tobacco plants. It is possible that the good effects of the catalytic fertilizers may be partly due to an acceleration of the enzyme activity in the plant.

For these experiments, iron and manganese salts of citric, malic and oxalic acids were used because it was thought that since these acids have been found in tobacco, their salts would not have a tendency to retard the growth of the plant. Besides the above substances, weak solutions of hydrocyanic acid and potassium cyanide were used in the series in two boxes to determine their effect on the plant. As emulsin has been found in tobacco, it was thought that the addition of these substances might prove interesting since it is generally recognized that small amounts of

⁸ Euler-Pope: *General Chemistry of the Enzymes*, 1912, pp. 108-10.

strong poisons sometimes act as powerful stimulants. Amygdalin was also tried alone in this series. As a continuation of the preceding series, mixtures of the substrate and the ferments were also included and finally another trial was made of the sulphuric acid treatment of the seed, except in this case the seed was soaked for only three minutes instead of fifteen as before.

The experiments were carried out in the same manner as those in the preceding series, except the seed was not soaked before planting. The same kind of soil prepared as previously described was used. Two hundred seeds, from the same lot used before, were sown in each box.

The materials used in the series were as follows:

- No. 1. Check.
- No. 2. Casein + trypsin + pepsin.
- No. 3. Pancreatin + peptone + glucose.
- No. 4. Sulphuric acid treatment of seeds.
- No. 5. Iron and manganese carbonate (Merck).
- No. 6. Iron and manganese peptonate (Eimer & Amend).
- No. 7. Manganese citrate (Merck).
- No. 8. Iron malate (Eimer & Amend).
- No. 9. Iron citrate, U. S. P. (Eimer & Amend).
- No. 10. Iron (ous) oxalate, pure (Eimer & Amend).
- No. 11. Potassium cyanide (Eimer & Amend).
- No. 12. Hydrocyanic acid (Eimer & Amend).
- No. 13. Iron and manganese lactate (Eimer & Amend).
- No. 14. Amygdalin.

The seeds were planted February 5, 1913, and the boxes were watered each time with 500 cc. of the following solutions made with hydrant water from the above substances. The substances given above which were used in the preceding series, were taken from the same samples used before.

- No. 1. 500 cc. hydrant water.
- No. 2. 500 cc. of a solution containing 0.0025 per cent of each substance.
- No. 3. 500 cc. of a solution containing 0.0025 per cent of each substance.
- No. 4. 500 cc. hydrant water.
- No. 5. 500 cc. of a 0.01 per cent solution.
- No. 6. 500 cc. of a 0.01 per cent solution.
- No. 7. 500 cc. of a 0.01 per cent solution.
- No. 8. 500 cc. of a 0.01 per cent solution.
- No. 9. 500 cc. of a 0.01 per cent solution.
- No. 10. 500 cc. of a 0.01 per cent solution.

No. 11. 500 cc. of a HCN solution containing 1.03 parts HCN per million.

No. 12. 500 cc. of a KCN solution containing 2.47 parts KCN, *i.e.*, 1.03 parts HCN per million.

No. 13. 500 cc. of a 0.01 per cent solution.

No. 14. 500 cc. of a 0.005 per cent solution.

In Nos. 2, 3, 11, 12 and 14 the solutions were made fresh each time, while in the others stock solutions of 2500 cc. were made as needed.

From the beginning, Nos. 3, 10, 11 and 12 were much better in appearance than the others and No. 10 had considerably more seeds to germinate in less time than the rest. Only a few seeds, not over eight or ten, germinated in No. 4 and this box was discarded.

On February 28, Nos. 3, 9, 10, 11, 12, 13 and 14 were better than the check but unfortunately some mice destroyed most of the large plants in these boxes, especially No. 10 in which the tops were gnawed close to the ground.

On March 6, the strengths of the HCN and KCN solutions were increased to 2.06 parts HCN per million and from this time about every three days, the strength was increased about 2 parts per million each time until on March 18, the solutions contained 10.3 parts HCN per million and these were used until the end of the experiments.

On March 24, the plants were transplanted and arranged in the boxes so as to evenly distribute them in the soil. At this time, in general appearance, No. 11 was in the lead; next in order were Nos. 6 and 12; then Nos. 3 and 13 were about the same; Nos. 2, 5, 7 and 10 were about like the check and Nos. 8, 9 and 14 appeared to be behind it.

On April 11, the plants in the best boxes were of sufficient size for transplanting. At this time they ranked in general appearance as follows: Nos. 6 and 11 were the best. The plants in No. 6 were more uniform and probably were a little better than No. 11, although the latter had some larger plants. Next in appearance were Nos. 3, 12 and 13. The plants in these boxes were not so very uniform especially in No. 12 which had some large plants and some very small ones. Nos. 6 and 13 had made good growth during the preceding few days. Next in order were

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Nos. 7 and 10 which were fairly uniform and looked slightly better than No. 1. Nos. 2 and 8 appeared about the same as the check. There were fewer plants in No. 8 but they were larger than those in No. 1. In No. 2 the plants were uniform while in the check they were not, which made the comparison very difficult. Nos. 5, 9 and 14 did not show up as well as the check.

On this date, the plants were cut close to the ground and weighed. For purposes of comparison, the weights of the green plants, the gain or loss in weight compared with the check, and the amounts of the different substances and nitrogen added in the same are given in Table II.

TABLE II.

NUMBER AND SUBSTANCE	WEIGHT OF PLANTS WHEN CUT	GAIN OR LOSS COMPARED WITH NO. 1	WEIGHT OF SUBSTANCE ADDED		NITROGEN IN SUBSTANCE ADDED		WEIGHT OF NITROGEN ADDED
	grams	per cent	grams	per cent	grams	per cent	gram
1. Check.....	95						
2. { Casein.....	92	-3.2	0.2625	14.64	0.0384		
Trypsin.....			0.2625	12.26	0.0322		
Pepsin.....			0.2625	5.14	0.0135		
3. { Pancreatin.....	114	+20.0	0.2625	12.58	0.0330		
Peptone.....			0.2625	15.88	0.0417		
Glucose.....			0.2625	0.64	0.0017		
4. Seeds treated with H ₂ SO ₄							
5. Iron and manganese carbonate...	69	-27.4	1.0500				
6. Iron and manganese peptonate...	142	+49.5	1.0500	10.32	0.1084		
7. Manganese citrate.....	91	-4.2	1.0500				
8. Iron malate.....	75	-21.1	1.0500				
9. Iron citrate.....	60	-36.8	1.0500				
10. Ferrous oxalate.....	82	-13.7	1.0500				
11. KCN.....	126	+32.6	0.1235	21.52*	0.0265		
12. HCN.....	108	+13.7	0.0512	51.85*	0.0265		
13. Iron and manganese lactate.....	105	+10.5	1.0500				
14. Amygdalin.....	62	-34.7	0.5250	3.06*	0.0161		

* Calculated.

CONCLUSIONS.

It is of interest to note in this series, which was carried on in the same manner and for the same time as the preceding one, that pepsin in combination with casein and trypsin failed to show

any increase of growth, whereas alone each substance before gave a decided gain. In No. 3, the combination of the three substances shows a material gain and each also gave an increase in the preceding series.

Of the other substances, iron and manganese peptonate, potassium cyanide, hydrocyanic acid and iron and manganese lactate gave positive results and in the order named. The remainder gave negative results and in some cases the losses were very large.

The results cannot be explained as due to the nitrogen added since some of the good boxes had much smaller amounts supplied to them than those in which the losses were very large. This becomes more apparent on comparing this and the former series in so far as pepsin is concerned.

The good results obtained with the potassium cyanide and hydrocyanic acid are worthy of further study and it would be interesting to find just what amounts of these substances the plant can stand in order to obtain its best growth and the effect, if any, they may have on its composition and quality.

In conclusion, the writers desire to express their appreciation to Dr. Joseph H. Kastle for suggesting a part of this work and for valuable advice given during its progress.



STUDIES ON THE THEORY OF DIABETES.

II. GLYCID AND ACETOLE IN THE NORMAL AND PHLORHIZINIZED ANIMAL.

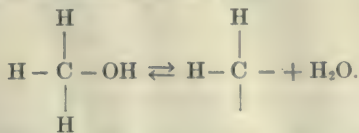
BY J. R. GREER, E. J. WITZEMANN AND R. T. WOODYATT.

(From the Otho S. A. Sprague Memorial Institute Laboratory of Clinical Research, Rush Medical College, Chicago.)

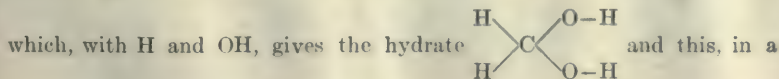
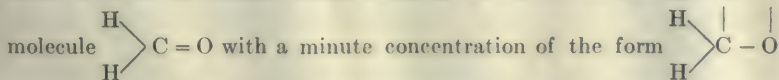
(Received for publication, October 27, 1913.)

Many organic chemical reactions are most simply explained by the theory that organic substances are in part dissociated to give residues which are in a state of dynamic chemical equilibrium with the undissociated molecules. Indeed for the explanation of an equilibrium such as that seen when sugars are dissolved in weak alkali some such theory is absolutely indispensable.

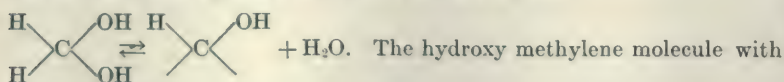
This conception has been developed elaborately by Nef according to whose view methyl alcohol, for instance, consists of a great preponderance of undissociated alcohol molecules in dynamic chemical equilibrium with a very minute quantity of methylene and water



The addition of an alkali like KOH leads to the formation of a salt CH_3OK (potassium alcoholate) the dissociation of which into KOH and methylene, is greater than that of alcohol into HOH and methylene, so that the effect of alkali is to raise the reactivity of methyl alcohol by increasing the concentration of methylene. Similarly, formaldehyde is held to consist under ordinary circumstances chiefly of the



manner analogous to that shown by methyl alcohol, dissociates with the loss of water into hydroxy methylene and water, thus:



an excess of available H becomes methyl alcohol; with an excess of OH (or O), formic acid; with H and OH in equal quantity it regenerates the aldehyde, etc. In short, *formaldehyde first dissociates and the fate of the dissociated particles depends upon the character of the reaction mixture in which they find themselves.* Alkali, heat, light, certain enzymes, etc., serve to increase dissociation.¹

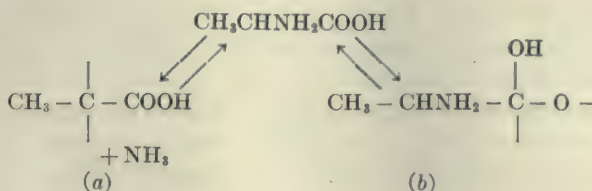
Multiplication of experiments makes it possible in many cases to recognize with considerable definiteness the actual configuration of the unsaturated residues concerned. Yet in the interpretation of metabolic phenomena this system of chemical thought has found little application. Ringer² holds for incorrect Neubauer's conception³ of oxidative deaminization of alanine and his belief that when alanine is broken down in the body, pyruvic acid is the main first "*fassbares*" product. This dissent is based on the fact that alanine in a fully phlorhizinized dog goes over almost quantitatively into sugar while pyruvic acid does so in some cases only to a limited extent if at all. But pyruvic acid, according to Embden, will yield lactic acid in a perfused liver and lactic acid will go over quantitatively into sugar in a phlorhizinized dog. The point which looms clearly out of these experiments would seem to us to be that alanine is first *dissociated* and that the dissociated residue may make lactic acid under conditions in which there is an equal concentration of OH and H, *i.e.*, when free O is deficient (Embden's perfusions), or form pyruvic acid where O is in excess and the whole bodily equilibrium is not upset in the direction of glucose (Neubauer's oxidative deaminization) or form glucose (via methyl glyoxal or lactic acid?) when the rapid withdrawal of glucose from the body upsets the entire chemical equilibrium of the metabolites in this particular direction (phlorhizin diabetes).

¹ Cf. *Liebig's Annalen*, cccxxxv, p. 191 *et seq.*

² *This Journal*, xv, pp. 145-52, 1913.

³ *Deutsch. Arch. f. klin. Med.*, xcv, p. 211.

One might assume, with a mass of chemical data to support the view, that alanine is dissociated in at least two ways, as follows:



Molecule *a* with H and OH gives lactic acid;⁴ with 2OH or O, pyruvic acid. Molecule *b* represents the phase which with an excess of H permits the reduction of the carboxyl group by addition of 2 H's and subsequent loss of H₂O, etc.

Again Embden assumes that because the trioses (*e.g.*, glyceric aldehyde) are capable of forming lactic acid by the action of surviving muscle, leucocytes, blood plasma, etc., that "Milchsäure ein auf dem Hauptwege des Traubenzuckerabbaus gelegenes Product sei."⁵ But all of Embden's experiments were carried out under what may be termed *asphyxial* conditions, and prove only that *when there is a lack of free oxygen* lactic acid is a chief product of the breakdown of the glucose. An *asphyxiated* alkaline glucose solution *in vitro* also forms lactic acid, but the latter is certainly no intermediate in the *fully oxygenized* alkaline glucose solution since no lactic acid appears in a fully aerated alkaline sugar solution, whereas preformed lactic acid is not destroyed when added to such a mixture. This experiment, performed by Meisenheimer and by Nef, we have amply confirmed. Glyceric aldehyde is rather first dissociated, and whether the residue burns or goes into lactic acid via methyl glyoxal will depend upon the conditions of the experiment.

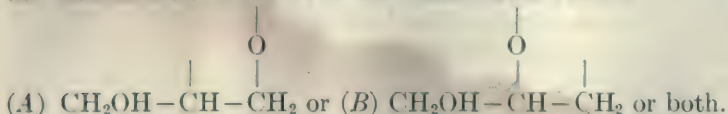
The following experiments with acetole and glycid were performed for the purpose of studying their behavior in the body apart from any theory but with special reference to the applicability of the above-mentioned views.

⁴ The simultaneous occurrence of types *a* and *b* with intramolecular rearrangement can yield methyl glyoxal—which by a benzilic acid rearrangement may give lactic acid.

⁵ *Biochem. Zeitschr.*, xlv, p. 108, 1912.

Glycid.

Glycid, $\text{CH}_2\text{OH}-\text{CH}-\text{CH}_2$, is an internal ether, representing a class of substances which, so far as we know, has not received attention from the biological standpoint, owing perhaps to the difficulties attending their preparation. Since the purely chemical behavior of these substances resembles that of the ordinary ethers, a parallelism might be anticipated in the body. Glycid does not reduce Fehling's or other sugar test solutions⁶ although it is generally stated to the contrary in the literature, and when boiled with two parts of water for eight hours, passes over into glycerol. This hydration implies a preliminary rupture of the ring with the intermediate existence of the unsaturated residue



Consequently if the ring in glycid were opened in the body, the biological effect of these residues might be determined. When glycid (or glycidic acid) is treated with halogen acids, the halogen is added chiefly to an end C atom, giving

$\text{CH}_2\text{OH}-\text{CHOH}-\text{CH}_2\text{Cl}$ (or $\text{COOH}-\text{CHOH}-\text{CH}_2\text{Cl}$) which is interpreted most simply by assuming that under these conditions the dissociation which yields the molecule *B* is the predominating one. Furthermore when glycid is heated (450°) it forms acetole $\text{CH}_2\text{OH}-\text{CO}-\text{CH}_3$ which is also ascribable to the

$$\begin{array}{c} \text{O} \qquad \qquad \qquad \text{O} \\ | \qquad \qquad \qquad || \\ \text{B residue, thus: } \text{CH}_2\text{OH}-\text{CH}-\text{CH}_2 \rightarrow \text{CH}_2\text{OH}-\text{C}-\text{CH}_3 \text{ (Nef).} \end{array}$$

If in the body the ring remained intact, the biological effects should resemble those of ethers in general. As a matter of fact the biological behavior of glycid is in harmony with the idea that the ring is but little attacked in the body.

Material. The glycid used was prepared by treating α -chlorhydrin with alcoholic KOH in the cold, and subsequent fractionation. It was a clear glycerol-like fluid of sp. gr. 1.10 (Westphal); b. p., 62°C . at 15 mm. pressure, and corresponding otherwise

⁶ Nef: *Liebig's Annalen*, cccxxxv, p. 232.

entirely with the product described by Nef.⁷ It had a slight aromatic odor and mild taste.

Animal experiments with glycid. Glycid was given subcutaneously to guinea pigs and rabbits. When 1 gram of glycid was given to a pig of 519 grams body weight (1.9 grams per kilo) no effects were at first apparent. After fifteen minutes the animal ceased feeding and became sluggish. In forty-five minutes it was very dull and could scarcely be aroused. There was slight twitching of the legs at this time, although it lay soporose in the natural position. In two hours it was dead without having changed its position except for the settling as the tone left the muscles. Post mortem examination showed an engorged right heart and lungs, a few subserous ecchymoses, the liver pale, with some fatty infiltration and parenchymatous degeneration, kidneys congested. Otherwise no change of note.

Dosages of 1 gram per kilo of weight were usually lethal. With 0.4 gram per kilo the animals became dull, but later recovered. In some fatal cases the parenchymatous changes in the liver and kidneys were more definite and the tendency to small hemorrhages greater so that the picture resembled chloroform poisoning. Death appeared to result from failure of respiration. Owing to the toxicity of glycid, no satisfactory results with phlorhizinized dogs were obtained.

The molecules *A* or *B* if formed in the body by opening of the ring, would be closely related to glycerol or acetole, neither of which is so highly toxic as glycid. These poisonous effects are accordingly ascribed to the preservation of the ring, *i.e.*, to the maintenance of the molecular form of an internal ether whose biological effects resemble those of other ethers.

Acetole.

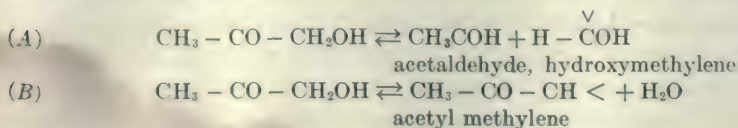
Acetole (hydroxy acetone) $\text{CH}_2\text{OH}-\text{CO}-\text{CH}_3$ stands between acetone on one side and the ketotriose, dihydroxy acetone, on the other. According to Emmerling and Loges,⁸ it is formed when hexoses are fused with caustic alkali and it has accordingly been held by certain writers to be an intermediate in the break-

⁷ *Loc. cit.*, p. 232.

⁸ *Ber. d. deutsch. chem. Gesellsch.*, xvi, p. 837.

down of sugars into lactic acid under the influence of alkali, and a similar rôle has been suggested for it in the body. It is the alcohol corresponding to pyruvic acid and methyl glyoxal (pyruvic aldehyde), concerning the former of which discussion is now in progress, and is isomeric with lactic aldehyde $\text{CH}_3\text{—CHOH—COH}$

and glycid $\text{CH}_2\text{OH—CH—CH}_2$. Acetole has not heretofore been directly tested in biological experiments. Its many known reactions are conveniently referable to two types of dissociation.⁹



The occurrence of the former type, *A*, is supported by the formation of acetaldehyde and metaformaldehyde when acetole is passed through heated tubes (at 450°) and by the formation of acetic and formic acids when acetole is oxidized with mercuric or silver oxide or with chromic and dilute sulphuric acids. The type *B* is suggested by Nef as the basis for the condensation of acetole and of analogous substances with strong alkali. Moreover when acetole is treated with copper acetate at 130° the copper salt is reduced and cuprous oxide is formed, the acetole passing over mainly into lactic acid. This lactic acid formation Nef ascribes to the formation from molecule *B* of acetyl formaldehyde (methyl glyoxal), $\text{CH}_3\text{CO—CH} < + \text{O} = \text{CH}_3\text{—CO—COH}$, which then by a benzilic acid rearrangement becomes lactic acid $\text{CH}_3\text{—CHOH—COOH}$. But when acetole is treated with alkali alone, no lactic acid, or only traces, occur. Consequently acetole cannot be an intermediate step in the formation of lactic acid by the action of alkali alone on hexoses. This lactic acid comes rather from methyl glyoxal by the process just mentioned—the latter substance being formed when alkali acts on hexoses *in the absence of oxygen*.

It follows from the foregoing that if a fundamental parallelism exists between the behavior of sugars in alkaline solution and in the body, as we have found it practical to assume, then acetole would not be an intermediate in the catabolism of glucose. Secondly, if the behavior of acetole itself in the body is analogous to

⁹ *Lisbig's Annalen*, cccxxxv, p. 250.

that observed in the presence of metallic oxides in weakly alkaline solution, its fate in the body should be chiefly that of acetaldehyde and hydroxy-methylene in accordance with the scheme A.

Now if acetole were convertible into lactic acid in the body by any process at all, the lactic acid so formed would be capable of yielding glucose in a fully phlorhizinized dog. This is equivalent to saying that if acetole by its dissociation could yield *B* particles which were convertible into lactic acid, acetole would be convertible into glucose. A failure on the part of acetole to form sugar would also speak against the dissociation *B* since this dissociation would yield methyl glyoxal (pyruvic aldehyde) which has been shown by Dakin and Dudley to be a sugar former.

The experiments with acetole warrant the conclusion that this substance is not a sugar former when given either by mouth or subcutaneously to phlorhizinized dogs, and not a normal intermediate between $C_6H_{12}O_6$ and $C_3H_6O_3$ (because all $C_3H_6O_3$ compounds are sugar formers regardless of their configuration; dihydroxy acetone [Mostkowski], glyceric aldehyde [Woodyatt], lactic acid [Lusk], hydracrylic acid). The results are most simply explained by assuming that acetole dissociates in the body in accordance with the scheme A, viz., $CH_3CO-CH_2OH \rightleftharpoons CH_3CO + \overset{\wedge}{CHO}$, correspond-

ing to that seen in the presence of weakly alkaline metallic oxides *in vitro*.

In the experiments in which acetole was first given to phlorhizinized dogs it was noticed that following its administration the urine gave the characteristic Gerhardt reaction with ferric chloride in dilutions twice as high as before. A definite increase was also noted in the difference between the polariscopic and titration estimations of sugar. This suggested an increase of the acetone bodies and in another experiment (II)—in which these were followed—the suspicion appears to have been confirmed, although the rising acidosis may have been a mere incident in the diabetes. An increase of acetone bodies may be attributed to aldole formed from acetaldehyde.¹⁰

Material. Acetole was prepared from bromacetone and sodium formiate in accordance with the method of Nef.¹¹ The product

¹⁰ Cf. A. Magnus-Levy: *Arch. f. exp. Path.*, xlv, p. 433, 1901.

¹¹ *Liebig's Annalen*, cccxxv, p. 260, *et seq.*

has a sweet taste and was colorless when freshly made but developed a straw color on standing. That used for the experiments was freshly distilled, and the portion used which passed over between 53° and 56° at 20 mm. pressure. For its detection in the urine we made use of the following properties: (a) volatility; (b) power to reduce metallic oxides in alkaline solution in the cold; (c) formation of a hydrazone (with phenylhydrazine); (d) lack of optical activity; (e) failure to give a color with Schiff's reagent for aldehydes.

Methods. Glucose in the urine was determined by polariscope and by the method of Bang and Bohmannsson, nitrogen by Kjeldahl; acetone and acetoacetic acid, Messinger; β -hydroxybutyric acid, Shaffer's method applied to the ether extract.

Phlorhizin was given as described in a previous paper.

Preliminary experiments with acetole. Healthy guinea pigs, rabbits and dogs received as high as 2 grams per kilo of body weight without fatalities. Nevertheless even 1 gram per kilo often produced symptoms. Following the ingestion or subcutaneous administration of acetole the urine becomes dark and contains then a little albumin and gives the characteristic absorption spectrum of haemoglobin. No reducing substance was found in the urine of a 10 kgm. dog after the administration of 20 grams of acetole by mouth, nor any other product of its decomposition. Acetole causes definite injury to the kidneys and this feature is also evident in the two following experiments.

EXPERIMENT I. Fully phlorhizinized fox terrier. Acetole 10 grams in 20 cc. of water made faintly alkaline with Na_2CO_3 administered subcutaneously at beginning of fourth 6-hour period.

PERIOD	DEXTROSE			N	D:N*	REMARKS
	Polariscope	Titration	Difference			
I	7.56	8.44	0.88	1.50	5.62	
II	6.20	7.20	1.00	1.95	3.77	
III	5.62	7.20	1.60	2.14	3.36	
IV	5.60	8.27 (7.27)	2.67 (1.67)	2.03	4.07	Urine dark, smoky with trace albumin.
V	4.31	5.31	1.00	1.41	3.76	Urine still darker, contains haemoglobin.

* D:N based on titration figures for dextrose.

The urine of period IV reduces Haines' solution in the cold. The cold reducing power measured by Bang's solution (one-half hour of contact) corresponds to 1 gram of dextrose. The ability of the urine to reduce in the cold is removed by boiling. The distillate contains a substance which reduces Haines' solution and silvers the walls of tubes containing ammoniacal silver oxide at room temperature. There is no reaction for aldehyde in this distillate with Schiff's reagent. With phenylhydrazine and paranitrophenylhydrazine, hydrazones were obtained from the distillate corresponding in appearance with those prepared for purpose of comparison from pure acetole. The extra reducing substance is therefore attributed to acetole.

EXPERIMENT II. Fully phlorhizinized fox terrier, weight 13 kg., which received 20 grams acetole dissolved in water, by mouth, at beginning of fifth 6-hour period.

PERIOD	DEXTROSE			N	D:N*	ACETONE AND ACETOACETIC ACID	β -HYDROXYBUTYRIC
	Polariscope	Titration	Difference				
I	11.78	43.75	1.97	1.86	7.39		
II	9.55	11.88	2.33	1.86	6.39	0.053	0.226
III	8.03	10.30	2.30	2.22	4.65	0.079	0.422
IV	6.00	8.55	2.55	2.28	3.75	0.132	0.951
V	4.65	7.50	2.85	1.96	3.83	0.275	0.997

* D:N based on titration figures for dextrose.

During the sixth period in this experiment the dog died. The urine after the administration of the acetole was smoky as usual and the distillate contained reducing substance which answered the reaction for acetole as in the previous experiment. Aldehyde was not demonstrable in the urine.

SUMMARY.

Glycid and acetole have been prepared in pure form and administered to healthy animals and to fully phlorhizinized dogs.

Glycid is toxic. Doses of 0.3 to 0.4 gram per kilo of body weight cause narcosis, accompanied at times by muscular twitching. Larger doses cause death. The effects are ascribed to the ring, which is opened in the body with difficulty.

Acetole is relatively non-toxic. Doses of 2 grams per kilo of body weight do not kill, but even moderate doses cause haematuria and haemoglobinuria. When given to phlorhizinized dogs either subcutaneously or by mouth, acetole causes no output of extra sugar. Some unchanged acetole may appear in the urine

and so raise its total reducing power. There is an apparent rise also of the acetone bodies. The behavior of acetole in the body is explained on the basis that it dissociates into acetaldehyde and hydroxy-methylene.

Acetole is not a normal intermediate between substances of the formula $C_6H_{12}O_6$ and those of the formula $C_3H_6O_3$.

THE IODINE CONTENT OF THE THYROID AND OF SOME BRANCHIAL CLEFT ORGANS.

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(From the Department of Physiology and Physiological Chemistry,
University of Manitoba, Winnipeg.)

(Received for publication, November 10, 1913.)

It is still a matter of uncertainty whether iodine is an invariable constituent of the thyroid gland, though all fresh evidence increases the probability that this is the case. Iodine is unquestionably present in the glands of most individuals and of most species. Numerous observers have confirmed its presence in the thyroids of man, cattle, sheep, swine, dogs, cats, and rabbits, and it has been observed further in the glands of stags, deer, goats, foxes, the stone-marten and pine-marten, guinea-pigs, hares, fowls, the African tortoise, the dogfish and skate.

In some instances negative results have been obtained. Baumann, who first observed its presence in the thyroid, obtained in man one negative case in ninety-one, in children twelve negative cases in thirty-nine, and in dogs two negative cases in nine.¹ Miwa and Stöltzner² stated that iodine is absent from the thyroids of normal new-born children. Roos³ obtained negative results in the case of three foxes, three out of four stone-martens, one of two pine-martens, one pole-cat, four of nine domestic cats, four of fourteen dogs, and three of seven pigs. Charrin and Bourcet⁴ obtained negative results with thyroids from eighteen of thirty-two children, and attributed the absence of iodine to their pathological condition. Mendel⁵ obtained four negative results from

¹ Baumann: *Zeitschr. f. physiol. Chem.*, xxii, pp. 1-17, 1896.

² Miwa and Stöltzner: *Jahrb. f. Kinderheilk.*, xlv, pp. 83-8, 1897.

³ Roos: *Zeitschr. f. physiol. Chem.*, xxviii, pp. 40-59, 1899.

⁴ Charrin and Bourcet: *Compt. rend. de l'Acad. des Sci.*, cxxx, pp. 945-8, 1900.

⁵ Mendel: *Amer. Journ. of Physiol.*, iii, pp. 285-90, 1900.

six children. Reid Hunt and Seidell⁶ obtained iodine-free thyroid from children, maltese kids, an Alaskan bear, and an aoudad.

In considering these negative results the analytical method must be taken into account. In all these cases the iodine was estimated by some variation of the method devised by Baumann,⁷ in which after fusion with sodium hydroxide, oxidation with nitrate, and subsequent dissolution, the iodine was extracted with chloroform, or carbon disulphide, and estimated colorimetrically. It has been shown by Seidell⁸ that this method frequently yields too low results, and it can therefore be inferred that minute quantities of iodine will frequently escape detection when it is employed. Hunt and Seidell⁹ in referring to the "negative" amounts in their material state explicitly that minute quantities may be present, but not detectable by their analytical method. *No stress can be laid on any negative results so far published until they have been confirmed by an accurate method such as that of Hunter.*¹⁰

In connection with the negative figures for children's thyroids, the results of Fenger,¹¹ who has employed Hunter's method, show clearly that iodine is normally present in the thyroids of foetuses and young of cattle, sheep, and swine, and in amount relatively comparable with that of thyroids in adult animals. It is probable that similar results will be obtained with children's thyroids when the same method is employed.

It is well recognized that there is marked variation in the iodine content of the thyroids of individuals of all species for which data have been obtained, but that in spite of this variation of individuals there is a definite relationship to diet exhibited. Thus the negative results quoted, even if they really indicate presence of slight amount of iodine, support Roos' assumption that the thyroids of carnivorous animals contain much less iodine than those of herbivorous species. Baumann's negative results with dogs

⁶ Hunt and Seidell: Bulletin No. 47, U. S. Hygienic Laboratory, p. 33, 1908.

⁷ Baumann and Roos: *Zeitschr. f. physiol. Chem.*, xxi, p. 489, 1896.

⁸ Seidell: *this Journal*, x, p. 95, 1911.

⁹ Hunt and Seidell: Bulletin No. 47, U. S. Hygienic Laboratory, p. 33, note (a).

¹⁰ Hunter: *this Journal*, vii, pp. 321-40, 1910.

¹¹ Fenger: *ibid.*, xi, pp. 489-92; xii, pp. 55-60, 1912; xiv, pp. 397-405, 1913.

were obtained after lean meat had been fed for some time, while it is now recognized that if any assimilable iodine compound be fed the iodine content of the thyroid is increased. There is strong presumptive evidence that the diet of all forms of sea-life is unusually rich in iodine, and I have recently shown that the thyroid of the dogfish (*Scyllium canicula*) contains an amount of iodine relatively greater than any previously recorded (1.16 per cent for dry material from female fish).¹²

It seemed desirable to extend the observations on iodine content to as many different classes of animals as possible, with the object of increasing the evidence both as to the invariable concomitance of iodine with thyroid tissue, and as to its variation in different classes consequent on their different diets. I have now obtained positive results with the thyroids of the pigeon, the alligator, the leopard frog (*Rana pipiens*) and a second species of dogfish (*Acanthias vulgaris*). The results for the pigeon are distinctly high, those for the alligator and frog distinctly low, in agreement with their respective herbivorous and carnivorous diets.

In addition some data are included bearing on the presence of iodine in parathyroid tissue. The sole results suggesting the presence of iodine in parathyroids in amounts comparable with that in thyroids are those of Gley.¹³ He found that in rabbits the absolute amount of iodine was greater in parathyroids; in dogs the relative amount was greater. He employed the Baumann method and any error in his results must apparently be attributed to the small quantities of materials employed. Chenu and Morel¹⁴ investigated dogs, rabbits, and fowls, comparing equal quantities of thyroid and parathyroid from the same animal. They concluded from their results that the parathyroid contains iodine, but to a less extent than the thyroid. Doyon and Chenu¹⁵ found that the parathyroids of the African tortoise contain little or no iodine. Estes and Cecil¹⁶ obtained negative results with the glands of the cow, horse, sheep, and man. Infinitesimal amounts present in

¹² Cameron: *Biochem. Journ.*, vii, pp. 466-70, 1913.

¹³ Gley: *Compt. rend. de l'Acad. des Sci.*, cxxv, pp. 312-5, 1897.

¹⁴ Chenu and Morel: *ibid.*, cxxxviii, pp. 1004-7, 1904.

¹⁵ Doyon and Chenu: *ibid.*, cxxxix, pp. 157-8, 1904.

¹⁶ Estes and Cecil: *Johns Hopkins Hospital Bulletin*, xviii, pp. 331-2, 1907.

two experiments with dogs' and one with horses' parathyroids were attributed to accidental thyroid contamination.

I have obtained absolutely negative results with the ventral branchial body of the frog—an organ whose function is still unknown. The presence of one or more parathyroids in close juxtaposition probably resulted in the removal of these organs with the ventral branchial body, so that the results may bear also on the iodine content of these organs.¹⁷ I have also carried out a comparison between the parathyroids and thyroids in a series of dogs. The results show at least a marked differentiation, while the actual amount found in the parathyroids may be completely attributable to the almost unavoidable contamination with thyroid tissue incident on the removal of the internal parathyroids in the dog. The results as far as they go support Estes and Cecil's conclusions that the parathyroids do not contain iodine.

I have used Hunter's method throughout. It has been tested and found accurate for ordinary amounts of iodine by numerous observers. I found some difficulty at first in obtaining perfectly negative results in known tests where iodine was absent, but found that, where the quantities of material analyzed were not greater than 0.5 gram, and after combustion, solution, and chlorination, the not-too-acid solution was boiled vigorously for at least one and one-half hours, the quantity of liquid being kept throughout between 150 and 300 cc., blank tests invariably gave perfectly negative results. Numerous tests with known quantities of iodine proved satisfactory. Hunter claims that his method will detect and approximately measure 0.01 mgm. of iodine (0.002 per cent of 0.5 gram). An absolutely negative result probably indicates a much lower iodine content than this figure.

The pigeon.

I have found only a single observation on the iodine content of the thyroid of birds. Chenu and Morel¹⁸ compared the thyroids

¹⁷ A full account of the anatomical relationships of these bodies and of the thyroid, for which the ventral branchial body must frequently have been mistaken, has been published recently by Mrs. F. D. Thompson: *Phil. Trans.* (B), cci, pp. 91-132, 1910.

¹⁸ *Loc. cit.*

and parathyroids of the domestic fowl. Since they took weights of thyroid tissue equal to those of the parathyroids present, only very small quantities were employed, and the results cannot be other than inaccurate. They are furthermore expressed for fresh tissue. This does not allow easy comparison with other published data. I have found that such small amounts of tissue can be weighed accurately only with difficulty on account of rapid drying. An approximation to the dry-tissue value may perhaps be obtained by dividing their results by four.

	WEIGHT OF THYROID TAKEN	AMOUNT OF IODINE FOUND	PER CENT IODINE	
			Fresh tissue	Dry tissue
	<i>gram</i>	<i>gram</i>		
1 year old cock.....	0.019	0.000011	0.058	(0.014)
1 year old cock.....	0.026	0.000014	0.054	(0.013)

It is doubtful whether these results show even the order of the amount of iodine present.

I have carried out a series of analyses with the domestic pigeon. Material was obtained from a large number of no certain type. In most cases the pigeons were less than six months old, the thymus being still well developed. The thyroid could be dissected completely from surrounding tissue, and the results are therefore probably correct to within 1 or 2 per cent (allowing for a trace of unremoved fibrous capsule). The material was dried *in vacuo* over sulphuric acid in this and all the succeeding analyses.

NUMBER OF THYROIDS TAKEN	WEIGHT		AMOUNT OF IODINE FOUND	PER CENT IODINE IN THE DRY GLAND
	Moist	Dry		
	<i>gram</i>	<i>gram</i>	<i>gram</i>	
23 (from 12 birds).....	0.236	0.075	0.000412	0.550
47 (from 24 birds).....	0.562	0.135	0.000644	0.477
36 (from 18 birds).....	0.504	0.117	0.000530	0.453
	Total, 0.327		0.001586	Average, 0.485

The alligator.

Three thyroids were obtained from three young alligators, each about twelve inches long. The dissection was clean, and the degree of error is determined only by the small amount of tissue examined.

WEIGHT OF DRIED THYROIDS	AMOUNT OF IODINE FOUND	PER CENT IODINE IN DRY TISSUE
gram	gram	
0.0402	0.0000239	0.059

These results agree with the low figures found generally for carnivorous animals.

The frog (Rana pipiens).

Treupel¹⁹ injected Baumann's iodothyryn subcutaneously into frogs, and in two cases removed the thyroids (under Gaupp's direction), and, employing Baumann's method, considered that he obtained unmistakable evidence of the presence of iodine in the tissue examined. This affords no evidence as to the presence of iodine under normal conditions, although Gaupp claims that it indicates that the thyroids of the frog function as in other vertebrates. Gaupp himself²⁰ states that he has confirmed the presence of thyroid tissue in the frog (*R. esculenta*, var. *Hungarica*) by a chemical test ("Die chemische Diagnose bestätigte, dass nicht irgend etwas Anderes, Muskelfasern und dergl. fälschlich für die Schilddrüse genommen war"), and since he immediately refers to Treupel's work he presumably indicates the presence of iodine, though I have found no further details of his examination.

I have examined the thyroids and ventral branchial bodies obtained from a large number of frogs (*Rana pipiens*) bought from Chicago and Minneapolis dealers during the period September to December, 1912, so that these frogs varied from well nourished to partially nourished individuals. On account of the minute size of the thyroid in the frog it is probable that some surrounding muscular tissue was frequently removed with it.

¹⁹ Treupel: *Münch. med. Wochenschr.*, xliii, pp. 885-6, 1896.

²⁰ Gaupp: cf. *Ecker-Wiedersheim's Anatomie des Frosches*, III, i, p. 206.

ORGAN	DRY WEIGHT	WEIGHT TAKEN	AMOUNT OF IODINE FOUND	PER CENT IODINE IN DRY TISSUE
	gram	gram	gram	
185 thyroids.....	0.2089	0.0987	0.0000727	0.073
		0.1102	0.0000592	0.054
		Total, 0.2089	0.0001319	Average, 0.063
188 ventral branchial bodies.....	0.2655	0.1169	0	
		0.1371	0	

The dry thyroid material was greasy as though some fatty tissue was present. In consequence sampling was difficult; this probably explains the non-agreement in the two results. They indicate definitely that iodine is present in the frog's thyroid under normal conditions. As has been mentioned, surrounding tissue was probably present, so that the figure must be regarded as a minimum one, to the extent perhaps of a from 20 to 40 per cent error. Even with this correction the amount present is distinctly small, corresponding with the known carnivorous habits of the frog.

The dogfish (Acanthias vulgaris).

As far as I am aware the only data for fish thyroids hitherto published are those I obtained this year for *Raia clavata* and *Scyllium canicula*.²¹ The samples of *Raia* gave figures varying from 0.283–0.438 per cent. A sample of male *Scyllium* thyroids gave the figure 0.719 per cent, another of thyroids from female *Scyllium* the very high figure 1.16 per cent.

Through the kindness of Professor E. E. Prince, Dominion Commissioner of Fisheries, a consignment of *Acanthias* was obtained last winter from the Atlantic Coast. They were preserved in formalin during transit, and it was found difficult to dissect the thyroid in the preserved fish, since the tissues had become hardened and discolored. In order to be certain that thyroid tissue was present much of the surrounding tissue was frequently removed, and the figure obtained consequently only indicates the order of the amount present. It was much smaller than that found for *Scyllium*. In

²¹ *Loc. cit.*

Iodine Content of Thyroid

all, 0.752 gram of material (dry) was obtained from a large number of fish.

WEIGHT TAKEN	AMOUNT OF IODINE FOUND	PER CENT IODINE IN DRY TISSUE
<i>gram</i>	<i>gram</i>	
0.2015	0.000271	0.134
0.1999	0.000263	0.131
		Average, 0.133

Comparison of the iodine content of the parathyroids and thyroids of the dog.

Twenty-three external and ten internal parathyroids were obtained from twelve dogs. They yielded 0.077 gram dry material which contained 0.0000120 gram iodine corresponding to 0.015 per cent. The thyroids were analyzed with the following results:

WEIGHT OF DOG	WEIGHT OF THYROIDS		AMOUNT OF THYROID TAKEN	AMOUNT OF IODINE FOUND	AMOUNT OF IODINE IN WHOLE GLAND	PER CENT IODINE IN DRY TISSUE
	Fresh	Dry				
<i>kgs.</i>	<i>grams</i>	<i>grams</i>	<i>gram</i>	<i>gram</i>	<i>gram</i>	
23.0	4.916	1.276	0.505	0.0000534	0.0001349	0
13.0	4.793	1.379	0.502	0.0007797	0.0021418	0
16.2	2.119	0.672	0.672	0.0017798	0.0017798	0
5.0	13.093	3.212	0.500	0.0004472	0.0028728	0
3.0	3.977	1.119	0.500	0.0009311	0.0020838	0
17.5	3.123	0.937	0.505	0.0005093	0.0009450	0
20.0	6.433	1.460	0.503	0.0004009	0.0011636	0
21.0	7.589	1.840	0.500	0.0000576	0.0002120	0
16.5	1.178	0.372	0.372	0.0006614	0.0006614	0
16.0	2.684	0.792	0.792	0.0005497	0.0005497	0
17.5	1.591	0.532	0.532	0.0001822	0.0001822	0
22.5	2.641	0.743	0.743	0.0009407	0.0009407	0
		Total, 14.334			Total, 0.0136677	Average, 0

The significance of this result has already been pointed out.

SUMMARY OF RESULTS.

Iodine is present in the thyroids of the pigeon, alligator, and frog, in amounts corresponding with the diets of these animals. It is also present in the thyroid of the dogfish (*Acanthias*). Fur-

ther support is therefore given to the theory that it is an invariable constituent of thyroid tissue. All reliable data hitherto published point to this invariable concomitance. The negative figures obtained by some investigators have led to some doubt as to the bearing of the presence of this element on the function of the gland,²² but it seems desirable to reject these negative figures entirely until more certain evidence is available.

Iodine is absent from the ventral branchial body of the frog.

The amount of iodine present in the parathyroids of the dog is of a less order of magnitude than that in the corresponding thyroids, if indeed the actual quantity observed be not wholly attributable to thyroid contamination. The results, so far as they go, indicate a differentiation of function between the thyroid and parathyroid.

Mrs. F. D. Thompson very kindly dissected the frog and alligator material for me, and, with Professor Vincent, dissected the dogfish. Professor Vincent dissected the dogparathyroids. My thanks are due to both for their very kind assistance in making this work possible.

The work forms part of a research conducted under the direction of the Committee on Ductless Glands of the British Association for the Advancement of Science. The expenses have been defrayed by grants from the British Association, and (to Professor Vincent) from the Government Grant Committee of the Royal Society of London.

²² Swale Vincent: *Internal Secretion and the Ductless Glands* (London, Arnold), 1912, p. 312 *et. seq.*



A GENERAL METHOD FOR THE CONVERSION OF FATTY ACIDS INTO THEIR LOWER HOMOLOGUES.

BY P. A. LEVENE AND C. J. WEST.

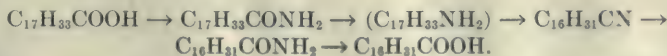
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Several general methods have been proposed for breaking down the carbon chain of the fatty acids, all of which are open to the objection either of poor yields or of lengthy and tedious procedure.

The first of these was developed by Krafft.¹ This consisted in distilling the barium salt of the C_n fatty acid with a slight excess of barium acetate in vacuum by which the methyl ketone $C_{n-1}COCH_3$ was formed. When this was carefully oxidized with potassium dichromate and dilute sulphuric acid, acetic acid was split off and the desired acid, $C_{n-1}H_{2n-2}O_2$, obtained.

A second method consists in the use of Hofmann's reaction.² In this the acid is changed into the acid amide, which is treated with three molecules of bromine and eight molecules of sodium hydroxide, giving the nitrile of the next lower fatty acid. This is then easily saponified to the acid amide and then to the acid. The steps are as follows:



Still another method has been published by Le Sueur³ and Blaise⁴ (the methods are the same, the interpretation of the course of the reaction different). The C_n acid is changed into the α -bromo-

¹ Krafft: *Ber. d. deutsch. chem. Gesellsch.*, xii, p. 1664, 1879.

² A. W. Hofmann: *ibid.*, xvii, pp. 1406 and 1920, 1884; E. Lutz: *ibid.*, xix, p. 1433, 1889.

³ Le Sueur: *Journ. Chem. Soc.*, lxxxv, p. 827, 1904; lxxxvii, p. 1888, 1905.

⁴ Blaise: *Compt. rend. de l'Acad. des. Sci.*, cxxxviii, p. 697, 1904; *Bull. soc. chim.* (3), xxxi, pp. 483-93, 1904.

and α -hydroxy-derivative, from which the aldehyde of the next lower acid is prepared by heating to 275° for about one hour, carbon monoxide being evolved during the heating. This aldehyde is then oxidized to the acid. Since the yield of the aldehyde is not over 50 per cent this method is not perfectly satisfactory, though Le Sueur claims it to be "a ready means of passing from an acid of the acetic acid series to the next lower homologue."

In certain cases, where the halide of the C_{n-2} alcohol is easily accessible, as is cetyl iodide, the C_{n-1} acid may be synthesized from the Grignard reagent and carbon dioxide.⁵

Edmed⁶ has observed that dihydroxystearic acid may be oxidized with alkaline permanganate at the place where the hydroxyls are attached. This method has been applied to the study of cerebronic acid,⁷ where it has been shown that the principal if not the only product of the reaction is lignoceric acid. It was then concluded to apply this method of oxidation on a larger number of α -hydroxy-fatty acids. It is comparatively an easy task to transform a fatty acid into its α -hydroxy-acid. If the permanganate method of oxidation were successful we hoped to apply the process for the study of the structure of lignoceric acid and of other fatty acids, the structure of which is not yet definitely established. Considerable time after the publication of the work on cerebronic acid Lapworth⁸ made use of the same process for the preparation of tridecyllic from myristic acid. Since then we have had occasion to prepare a considerable quantity of margaric acid and have applied the reaction to α -hydroxystearic acid with equal success. It has further been tried out in the preparation of pentadecyllic acid and we now believe that it is a general method for the decomposition of the carbon chain which may be easily carried out with fairly good yields. We obtained a yield of 80-85 per cent of margaric acid, calculated on the stearic acid used. It has the advantage over Le Sueur's method in that the preparation of the aldehyde with its consequent loss is avoided.

⁵ Ruttan: *Eighth International Congress of Applied Chemistry*, xxv, p. 431; *Chem. Abstracts*, vii, p. 2140, 1913.

⁶ Edmed: *Journ. Chem. Soc.*, lxxiii, p. 627, 1898.

⁷ Levene and Jacobs: *this Journal*, xii, p. 381, 1912; Levene and West: *ibid.*, xiv, p. 257, 1913.

⁸ Lapworth: *Journ. Chem. Soc.*, ciii, p. 1020, 1913.

In agreement with Lapworth we find that the reaction is best carried out in acetone rather than in water. The potassium salt of the new fatty acid (especially of the higher acids) is insoluble in acetone and precipitates with the manganese dioxide, from which it is easily extracted with alcohol.

EXPERIMENTAL PART.

Lignoceric acid.

The preparation of lignoceric acid has been modified to the following: Fifty grams of cerebronic acid are dissolved in about 1 liter of boiling acetone and this solution treated gradually with a warm acetone solution of potassium permanganate until the solution is slightly colored, indicating an excess of permanganate. The mixture is then heated a short time on the water bath, cooled, the manganese dioxide and potassium lignocerate and cerobronate filtered off, and the potassium salt extracted with boiling absolute alcohol. Usually two or three extractions, using a liter of alcohol each time, is sufficient. The acid thus obtained is converted at once into the lithium salt and purified with methyl alcohol as before. The acid from the insoluble lithium salt was recrystallized from acetone, when it gave the following numbers on analysis:

0.1232 gram of substance gave 0.3537 gram CO_2 and 0.1460 gram H_2O .

	Calculated for $\text{C}_{24}\text{H}_{48}\text{O}_2$	Found:
C	78.20	78.30
H	13.20	13.26

Margaric acid.

Stearic acid was converted into α -bromostearic acid by Hell's method and this into α -hydroxystearic acid according to Le Sueur. This was characterized by the preparation of

α -Acetoxystearic acid.

Fifteen grams of hydroxystearic acid were dissolved in 100 grams of acetyl chloride and the solution heated an hour in a water bath. The excess of acetyl chloride was removed on a boiling water bath, the product treated with water to remove the last

traces of the chloride and hydrochloric acid, extracted with ether, the ethereal solution dried, the ether removed and the product recrystallized from a little absolute alcohol. It is a colorless crystalline body which melts at 70–70.5°.

0.1258 gram of the substance gave 0.3243 gram CO_2 and 0.1258 gram H_2O .

	Calculated for $\text{C}_{20}\text{H}_{38}\text{O}_2$	Found:
C.....	70.12	70.31
H.....	11.19	11.19

Oxidized with potassium permanganate in acetone as given above, the hydroxystearic acid gave nearly pure margaric acid, which was purified by two recrystallizations out of acetone. It melted at 59–60°, as given by Ruttan.⁹ Its purity was controlled by analysis:

0.1228 gram of the substance gave 0.3386 gram CO_2 and 0.1360 gram H_2O .

1.0000 gram of the acid, dissolved in absolute alcohol and benzene required 37.0 cc. $\frac{\text{N}}{10}$ NaOH for neutralization, using phenolphthalein as an indicator.

	Calculated for $\text{C}_{17}\text{H}_{32}\text{O}_2$	Found:
C.....	75.60	75.20
H.....	12.60	12.40
M.W.....	270	270

Pentadecylic acid.

Pentadecylic acid was prepared in the same manner, starting with palmitic acid. The acid thus prepared melted at 53° and gave the following analytical figures.

I. 0.1342 gram of the substance gave 0.3674 gram CO_2 (H_2O lost).

II. 0.1324 gram of the substance gave 0.3620 gram CO_2 and 0.1472 gram H_2O .

1.0000 gram of the acid, as above, required 41.4 cc. $\frac{\text{N}}{10}$ NaOH for neutralization.

	Calculated for $\text{C}_{15}\text{H}_{30}\text{O}_2$	Found:	
		I	II
C.....	74.40	74.67	74.57
H.....	12.40		12.44
M.W.....	242	241	

⁹ Ruttan: *loc. cit.*

AUTOLYSIS OF MOLD CULTURES II.

INFLUENCE OF EXHAUSTION OF THE MEDIUM UPON THE RATE OF AUTOLYSIS OF *ASPERGILLUS NIGER*.

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In a previous paper¹ it was shown that when molds are grown upon a fluid synthetic medium, the nitrogen is almost completely taken up by the mycelium during the vegetative period of the fungus and then gradually returned to the medium after the growth has come to a standstill. In the particular medium studied where nitrogen and sucrose were present in the proportion of 1 to 50, most of the nitrogen had disappeared at the end of the first week, and during the subsequent seven or eight weeks a large part of it reappeared, principally in the form of ammonium salts. During this time the mycelium lost its turgidity and the medium became dark in color although it retained its original clarity. This phenomenon was ascribed to autolysis of the fungus.

The changes observed both in the mycelium and in the medium were so striking as to be deemed worthy of further study. Subsequent observations of numerous cultures showed that the rate of autolysis, as indicated by the visual appearance of the mycelium and of the medium, was influenced by a number of factors, such as the volume of the medium as compared with the surface area, the ratio of carbon to nitrogen, the temperature to which the cultures were exposed, etc. In other words, the supply of nutriment and the rate of growth seemed to be of primary importance in determining the point at which autolysis set in. As long as the presence of sugar could be demonstrated by Fehling's test, autolysis was scarcely noticeable.

¹ Dox and Maynard: this *Journal*, xii, pp. 227-31, 1912.

The experiments herein described were made for the purpose of studying the effect of exhaustion of the carbohydrate in the medium upon the autolysis of the fungus.

In the first series of cultures the following medium was used: distilled water 1000 cc., sucrose 30 grams, dibasic potassium phosphate 1.0 gram, magnesium sulphate 0.3 gram, ammonium acid tartrate 4.0 grams, trace of ferrous sulphate. Two hundred cc. of this medium were placed in each of a number of liter Erlenmeyer flasks, sterilized in an autoclave, and inoculated with spores of *Aspergillus niger*. At the end of a week vigorous cultures with an abundance of black spores were obtained. Two of the cultures were treated as follows: The cotton plug was removed, a sterile glass siphon inserted and the plug replaced. The medium was drawn off by suction, replaced by 200 cc. of sterile distilled water, and the latter removed in the same way after a few moments' contact with the mycelium. This was in turn replaced by 200 cc. of sterile water in one flask and by 200 cc. of a sterile 2 per cent sucrose solution in the other flask, and the cultures allowed to stand another week. This operation was repeated at the end of each week for six successive weeks. All this was done with as little injury as possible to the mycelium, care being taken not to wet the surface of the culture. Five other flasks were treated in the same manner after the cultures had grown two, three, four, five and six weeks respectively, except that the cultures were discarded after the medium and wash water had been obtained. The combined

TABLE I.

AGE OF CULTURE	a.		b.		c.
	MEDIUM REPLACED WEEKLY BY WATER		MEDIUM REPLACED WEEKLY BY 2% SUCROSE		MEDIUM FROM UNDIS- TURBED CULTURE
	mgm. N in medium		mgm. N. in medium		mgm. N
weeks		total		total	
0	70.0		70.0		70.0
1	2.7	2.7	2.5	2.5	2.4
2	12.4	15.1	3.1	5.6	9.4
3	12.8	27.9	2.3	7.9	16.9
4	5.9	33.8	1.4	9.3	23.5
5	3.6	37.4	1.0	10.3	26.1
6	3.0	40.4	1.7	12.0	27.3

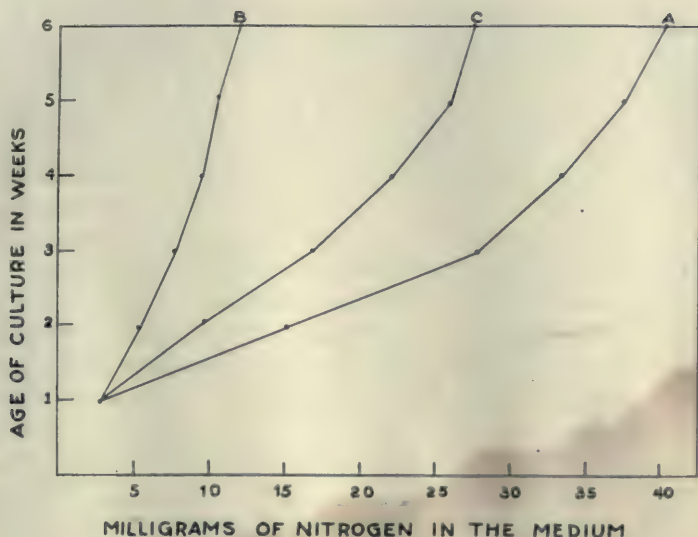


FIG. 1.

medium and wash water were used in each case for the determination of total nitrogen by the Kjeldahl method. As the liquid was not filtered it contained a few of the submerged mycelial filaments, but the nitrogen content of these was so small as to be negligible. This method of obtaining the medium does not allow for the liquid still retained between the closely matted hyphae, but no better method presented itself which would not be apt to injure the mycelium and disturb the normal progress of growth and autolysis of the organism. The results of the nitrogen determinations are given above.

The above experiments were repeated using Raulin's well-known medium, on which this organism grows still more luxuriantly. At the end of a week dense white mycelia were obtained, with spores just beginning to show around the edges. An abundance of black spores appeared about two days later. The sucrose solution used in this series was 4.67 per cent corresponding to the concentration of the same in Raulin's medium.

In both series the nitrogen restored to the medium in six weeks is more than three times as great when the medium is replaced weekly by distilled water as when it is replaced by sucrose solution.

TABLE II.

AGE OF CULTURE	MEDIUM REPLACED WEEKLY BY WATER		MEDIUM REPLACED WEEKLY BY 2% SUCROSE	
	mgm. N in medium		mgm. N in medium	
<i>weeks</i>		total		total
0	225.3		225.3	
1	17.5	17.5	18.3	18.3
2	48.2	65.7	8.3	26.6
3	46.2	111.9	8.5	35.1
4	20.0	131.9	4.9	40.0
5	14.3	146.2	4.5	44.5
6	4.5	150.7	3.8	48.3

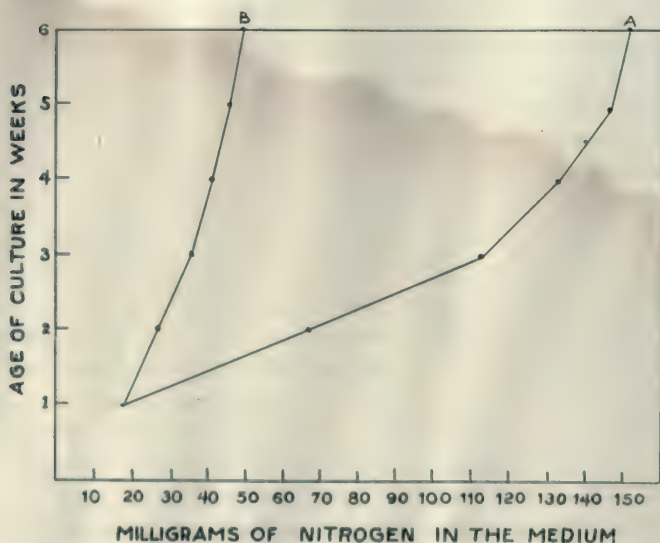


FIG. 2.

Other differences are equally striking. In the one case the medium is dark brown in color and neutral to litmus each time it is drawn off, in the other case it is pale yellow and strongly acid. The mycelium on water becomes thin and limp, while that on sucrose becomes more and more dense and retains its turgidity. It is evident that vegetative growth continues for a much longer period when sugar is supplied from time to time, although no nitrogen or inorganic

salts are added. Even after autolysis has been in progress for five weeks, the substitution of sucrose solution for the dark colored medium results in a marked decrease in the amount of nitrogen liberated. During the sixth week a five-week-old culture that had not previously been disturbed liberated only 10.4 mgm. nitrogen into the sucrose solution. A parallel culture liberated 48.2 mgm. nitrogen into distilled water.

The first series (Table*1) shows that the rate of autolysis is increased by removing the products of autolysis, as will readily be seen by comparing column *c* with the totals in column *a*. Where the autolytic products are not removed, the nitrogen determinations take a position on the curve intermediate between those occupied by the nitrogen in the sucrose medium and the nitrogen in the water.

The color of the medium may be taken as an indication of the extent of autolysis. Invariably the intensity of the brown color was proportional to the amount of nitrogen in solution.

When the medium is replaced from week to week by Raulin's medium instead of the sucrose solution, the greater part of the nitrogen continues to be assimilated, and the resulting mycelium is even more dense than that from sucrose alone. At the end of six weeks the mold was removed only with difficulty from the flask. On the other hand, the addition of an antiseptic appears to increase the rate of autolysis. A culture one week old, when floated upon 200 cc. of a dilute solution of mercuric chloride ($\frac{M}{1000}$), yielded 136.2 mgm. of soluble nitrogen as compared with 48.8 mgm. and 8.3 mgm. in the parallel experiments with water and sucrose respectively. However, the effect of the antiseptic after spore production is much less pronounced.

During autolysis the weight of the mycelium decreases while the percentage of nitrogen in the latter remains fairly constant. This is clearly indicated by the following data, where the mycelium from five cultures of *Aspergillus fumigatus* was removed each week, pressed as free as possible from the medium and dried in an oven.

The weights in the following table were not expressed more accurately for the reason that the autolyzed mycelium becomes pasty when subjected to pressure and cannot be removed completely from the cloth. The probable loss from this cause was about 1 per cent. The data are sufficiently accurate, however, to show

TABLE III.

AGE OF CULTURE	DRY WEIGHT OF MYCELIUM	NITROGEN IN MYCELIUM	
weeks	grams	per cent	gram
3	11.5	6.14	0.706
4	10.0	6.24	0.624
5	9.0	5.78	0.520
6	8.5	5.73	0.487
7	7.9	5.68	0.500
8	7.5	6.08	0.456
9	7.4	5.62	0.416
10	7.0	5.52	0.386
13	6.5	5.66	0.368

the gradual loss in weight when the mycelium undergoes autolysis, while the percentage of nitrogen scarcely changes. The weights for the first and second weeks were not obtained, but it is probable that the loss at the end of thirteen weeks is at least 50 per cent of the maximum weight on the dry basis.

The more important observations recorded in this paper may be summarized as follows:

1. Autolysis of cultures of *Aspergillus niger* is due chiefly to exhaustion of carbohydrate from the culture medium.

2. The rate of autolysis is increased by removing the autolytic products and replacing by distilled water.

3. Replacement of the medium at regular intervals by a sucrose solution reduces the rate of autolysis to less than half that of the undisturbed culture, and less than one-third that of the cultures where the medium is replaced by distilled water.

4. Autolysis is attended by a loss in weight of the mycelium amounting to about 50 per cent in thirteen weeks.

My thanks are due to Mr. W. G. Gaessler who kindly made the nitrogen determinations.

CARBON DIOXIDE APPARATUS III.¹

ANOTHER SPECIAL APPARATUS FOR THE ESTIMATION OF VERY MINUTE QUANTITIES OF CARBON DIOXIDE.

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(Received for publication, November 22, 1913.)

For the purpose of estimating a very small amount of carbon dioxide in general biological problems, two pieces of apparatus have been already described.² The principle of the apparatus is as follows:

1. Exceedingly minute quantities of carbon dioxide can be precipitated as barium carbonate on the surface of a small drop of barium hydroxide solution.

2. When a drop of barium hydroxide is exposed to any sample of gas free from carbon dioxide it remains perfectly clear, but when more than a quite definite minimum amount of carbon dioxide is introduced a precipitate of carbonate appears, detectable with a lens.

3. By determining, therefore, the minimum volume of any given sample of a gas necessary to give the first visible formation of the precipitate, its carbon dioxide content can be estimated accurately, since this volume must contain just the known detectable amount of carbon dioxide.

In order to determine this minimum volume of the gas in the respiratory chamber, it has been recommended that in the case of biological problems, when the specimen gives off carbon dioxide continuously, and sometimes at different rates, varying with the time, it would be much simpler not to attempt to determine the minimum

¹ This and my other apparatus can be obtained from Eimer and Amend, New York.

² *Amer. Journ. of Physiol.*, xxxii, p. 137, 1913.

volume by a continuous trial with the same sample of tissue, but instead to repeat the experiments with a series of different samples of known weights for a known time, and determine the minimum volumes which give the precipitates, and the maximum volumes which do not give the precipitates.³ In this way, one can easily calculate what is the minimum volume which gives the precipitate, for a given weight of the specimen for a given time. All of the analyses of the gas in connection with metabolic problems of the nerve fiber have been done in this way with satisfactory accuracy.

Although the use of the biometer (apparatus II) is perfectly satisfactory for almost all micrometabolic problems, and sometimes absolutely necessary for quick quantitative comparisons between two different tissues, yet it is sometimes inconvenient for a complete determination of the CO_2 -production from a single tissue, the metabolic rate of which is constantly changing, and the available amount of which is not very great. The necessity for a device to prevent this difficulty was keenly felt when I was studying the metabolism before, after and during the cleavage of a single fertilized egg this summer. The new apparatus III, here reported, proved to be indispensable for such an investigation and may be of some interest for general biological problems, for which the previous apparatuses are found to be useful.

The new feature of this apparatus III is a device by which the air can be withdrawn into a tube from the respiratory chamber and can be analyzed subsequently. With this device, one can not only make a complete analysis with one sample of the tissue, but can also make several complete estimations with it. The detailed method will make this clear.

1. *The apparatus.*

As shown in figure 1, the main part of this apparatus consists of only one glass bulb *A*, which serves the combined purpose of respiratory and analytic chambers. Its volume, originally 30–40 cc., can be diminished by introducing mercury in exactly the same manner as described previously. Similarly, the $\text{Ba}(\text{OH})_2$ tube *d* is inserted through its wall, and a three-way stopcock *4* is attached to the bottom of the chamber. Just opposite the top of

³ See a footnote on p. 140, *loc. cit.*

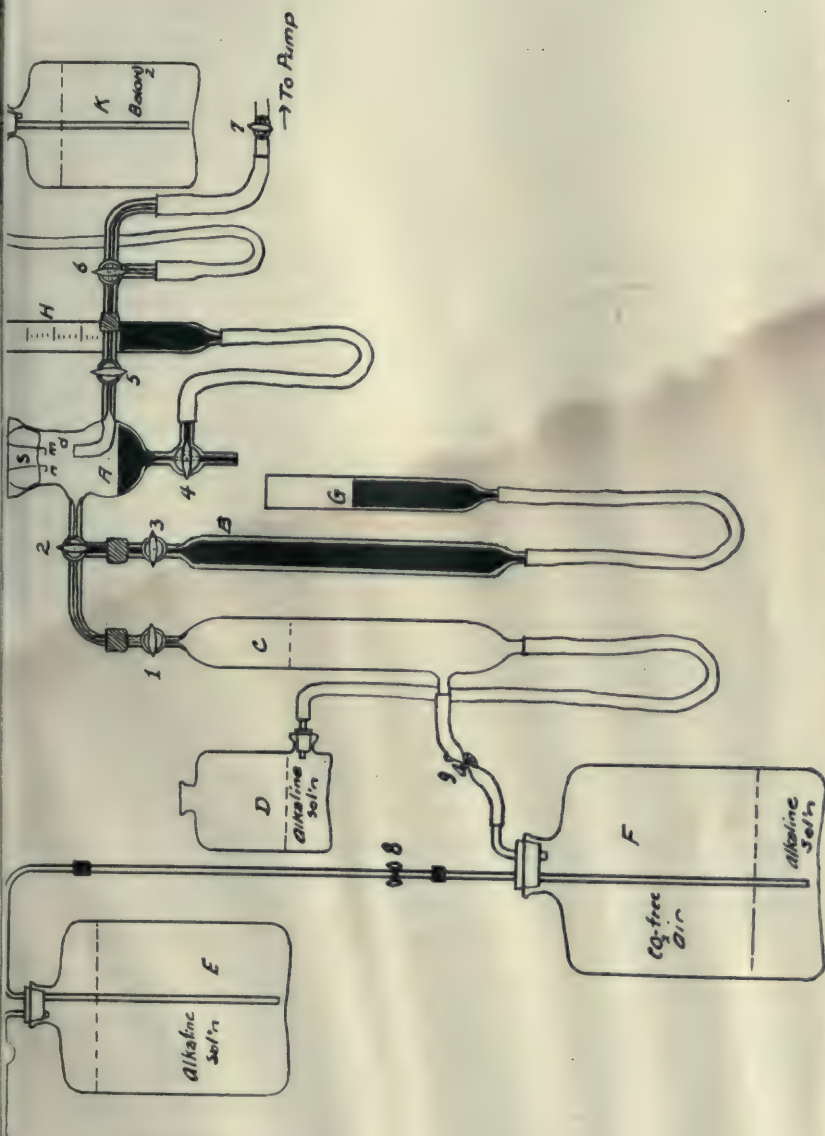


FIG. 1. A simpler apparatus for CO_2 analysis, showing also the device to obtain air free from CO_2 ; $\frac{1}{4}$ actual size except E and F which are $\frac{1}{16}$.

the $\text{Ba}(\text{OH})_2$ tube *d*, another three-way stopcock 2 is inserted, one arm of which is connected to the nitrometer *C*, and the other to tube *B*. This tube *B* is attached to the mercury burette *G*, by which the pressure in the tube is to be adjusted. A similar mercury burette is also attached to one arm of three-way stopcock 4 for the same purpose.⁴

2. *How to obtain CO_2 -free air.*

Several inquiries have been made as to the exact arrangements for obtaining CO_2 -free air, and how to use them. As I have stated elsewhere, it is very difficult to make air completely free from carbon dioxide by merely passing it through alkaline wash bottles. A simpler and sure method to obtain CO_2 -free air is shown in fig. 1. It is prepared by shaking air with a 20 per cent solution of sodium hydroxide in a tightly stoppered carboy *F*, supplied with suitable tubes. When this air is to be used it is driven into a nitrometer *C*, which is filled with less concentrated alkaline solution (a weak solution is used so that the chamber may not be too dry), by displacing it by running into *F* a solution of sodium hydroxide with a funnel, or from another carboy *E* which is filled with the alkali. After each evacuation of the chamber, this air is introduced from the nitrometer *C* into the chamber *A* through stopcock 1. For ordinary experiments, one can always keep the pressure in *F* high enough so that CO_2 -free air may be driven several successive times into nitrometer *C* by simply opening pinchcock 9.

In order to test whether or not the air thus treated is now free from carbon dioxide, the following experiment will be necessary.

Remove the glass stopper *S*, and introduce into the chamber a known amount of mercury by means of the mercury burette *H*, so that the remaining volume of the chamber *A* is exactly known.⁵

⁴ Instead of fusing the platinum electrodes into the wall of the chamber, they were fused into the glass stopper *S*. It will be clear by inspection of the figure that the wires are brought up high enough so that when the chamber is sealed with mercury there will be no short circuit established through mercury and electrodes, in case any electric current is used for the stimulation of the tissue.

⁵ The exact capacity of chamber *A* should be calibrated once for all. In this way, one can always work with a constant volume in the chamber by

Turn stopcock 4 now about 45° , so that the connection between *A* and *H* is severed. Replace the stopper *S*, and seal the chamber with mercury. Keep stopcock 2 turned so that the connection is made only between nitrometer *C* and chamber *A*. The alkali in the nitrometer *C* is displaced by CO_2 -free air by opening pinchcock 9. Collect about 300 cc. of the CO_2 -free air in the nitrometer *C*. While the stopcock 1 is closed, the chamber *A* is evacuated by means of suction, having the stopcocks 5, 6 and 7 opened (the three-way stopcock 6 should be opened in such a way that $\text{Ba}(\text{OH})_2$ is completely shut off from the connection).

When the evacuation is complete, CO_2 -free air is introduced into the chamber by opening stopcock 1. After the evacuation and washing out with pure air, which is repeated four or five times, the chamber now being filled with CO_2 -free air, the stopcock 7 is closed, and the pressure inside chamber *A* is made equal to the atmospheric pressure by adjusting it at the nitrometer *C* by means of the alkali bottle *D*. Stopcock 5 is then closed, and the space between 5 and 7 is again evacuated so that the barium hydroxide can rush in, a process which is very advantageous in obtaining a clear barium hydroxide solution. In filling the tube with the barium hydroxide, it is advisable to open stopcock 6 so that the solution will first fill up the space between 6 and 7, then turn it in such a way that now the connection is made between the barium hydroxide tube and the space between stopcocks 5 and 6. By opening 5 very slowly and carefully, the barium hydroxide is now introduced into the chamber just so far that a small hemispherical drop stands upon the upturned end of the tube at *d*. After quickly readjusting the pressure by means of the nitrometer and the bottle *D*, the stopcock 1 is closed. If the air is completely free from carbon dioxide, the drop should be clear not only at the start, but also, after several hours' standing, free from any granules of the carbonate, when inspected with the lens.

introducing the necessary odd cubic centimeters of mercury, thus making the remaining volume a convenient round number of cubic centimeters. For instance, the apparatus I am using has a capacity of 31.4 cc. I introduced 6.4 cc. of mercury for each experiment, so that the respiratory chamber then contains 25 cc. It will be needless to say that for an experiment to test the air for its purity, the knowledge of the exact capacity of the chamber is not at all necessary.

3. For the qualitative detection of carbon dioxide.

For the detection of carbon dioxide production from a tissue, this apparatus can be used in exactly the same manner as the previous apparatus. After insuring that the air is free from the gas a given tissue⁶ is placed inside of the chamber and the process is repeated as before. If any CO_2 is given off by the tissue not only will a deposit of carbonate appear, but it will also grow in size.

4. For quantitative estimation of the gas.

The detailed method is as follows.

Open stopcocks 3 and 2 so that they will connect the chamber *A* and the tube *B* only. Fill the mercury burette *G* and raise it till the mercury will completely fill the tube *B* and a little excess of it will stay in the capillary tube between the chamber *A* and the stopcock 2. This stopcock 2 is now closed so that it will connect the nitrometer *C* and the chamber *A* only. Increase the pressure inside the nitrometer *C* by raising the alkali bottle *D* much higher than the meniscus of the alkali in the nitrometer *C* and then open stopcock 1. In this way, the excess of mercury left in the capillary tube will be pushed over into the chamber and will flow through the stopcock 4 into a receiving vessel.

If the stopcock 2 is absolutely air-tight, having no air bubble in tube 3, then a known amount of mercury is introduced into the chamber by means of the mercury burette *H*, thus giving the respiratory chamber the desired volume. The tissue is introduced into the chamber, the glass stopper is replaced, the chamber is sealed with mercury, and the nitrometer *C* is filled with the pure air. After evacuation of the chamber and washing it with CO_2 -free air several times, the stopcock 5 is closed and the time is recorded; the pressure is adjusted, and stopcock 2 is turned 45° .

At the end of the desired respiration period, any portion of the air in the chamber can be driven into the tube *B*. This is accomplished by raising the right hand mercury burette *H*, and simultaneously opening the stopcocks 2 and 4. Stopcock 2 is now

⁶ The tissue can be placed on a cover slide and allowed to float on top of the mercury or it can be placed on the glass plate and hung on the electrodes as described in figure 1, p. 120, *loc. cit.*

closed: the pressure of the air in *B* is kept equal to the atmospheric pressure by adjusting the mercury burette *G*. After removing the mercury seal and glass stopper *S*, the tissue is withdrawn and mercury burette *H* is lowered so that most of the mercury in the chamber will now flow back into the burette *H*. The excess of mercury in the chamber *A* is withdrawn through stopcock 4 into a vessel.

In order to analyze the air in the tube *B*, it is advisable to clean the whole chamber *A* once more with water,⁷ and then to perform the experiment in exactly the same manner as we described in connection with the test for purity of air.⁸ Two things are imperative, namely; the capacity of the respiratory chamber *A* must be known exactly after the known amount of mercury is introduced into it; and the bubble of barium hydroxide solution at *d* must be perfectly clear at the start. If no deposit of barium carbonate forms on the surface of the drop within ten or fifteen minutes, it is a sure control that the apparatus is free from carbon dioxide. This point established, a portion of the sample of gas in the tube *B* is introduced into the chamber. This is very easily accomplished by withdrawing the mercury from the chamber *A* into a small graduated cylinder⁹ and adjusting the pressure by raising the left-hand mercury burette *G*; then close the stopcock 2 by turning it 45°.

One now watches the surface of the drop at *d* with a lens to see whether any formation of barium carbonate occurs within ten minutes. If it does not, we should introduce more air from *B* until we get the first visible precipitate.¹⁰ I have previously determined,¹¹ by introducing accurately known quantities of carbon dioxide of very high dilution into the chamber in a similar manner, and have found with remarkable regularity that 1×10^{-7} gram of carbon dioxide is necessary as the minimum amount to give a

⁷ For the method of cleaning this apparatus and drying it in ten minutes without taking it apart, see a footnote on page 138, *Amer. Journ. of Physiol.*, xxxii, 1913.

⁸ See pages 488-89.

⁹ If more accurate measurement is necessary, the mercury withdrawn should be weighed.

¹⁰ The detection of this precipitate is not a question of degree, but is a question of the appearance of some precipitate or none at all; therefore the end point is very sharp.

¹¹ See p. 144, *loc. cit.*

precipitate within ten minutes.¹² Smaller amounts of the gas give no visible results, while larger amounts give a deposit more rapidly and in larger quantities. This minimum detectable amount of 1×10^{-7} gram is about the amount which is contained in 0.17 cc. of natural air in which we assume 3 parts of carbon dioxide in 10,000 by volume. The following example will illustrate the calculation of the exact amount of the gas a tissue gives off.

The original volume of the respiratory chamber is 31.4 cc., to which 6.4 cc. of mercury are introduced, making the remaining volume exactly 25 cc. 10 mgms. of tissue are used and are allowed to respire in the chamber for ten minutes. Then about 10–15 cc. of the gas are withdrawn into the tube *B*. 0.5 cc. of this gas gave no precipitate during the first ten minutes; 0.5 cc. more of the sample gave no deposit in another interval of ten minutes; 0.5 cc. more, a total of 1.5 cc., was run into the chamber. A marked evidence of a precipitate appeared in five minutes. 1.5 cc. of this gas must therefore contain 1×10^{-7} gram of carbon dioxide. The apparatus is then cleaned and dried and a clear drop of barium hydroxide is again introduced upon the top of the tube *d*; and after again insuring the fact that the air is free from any CO₂, by waiting, 1 cc. of the sample gas which has been left undisturbed in tube *B* is introduced into the chamber; no precipitate was formed within ten minutes; 0.25 cc. more of the sample did not produce any precipitate; but when 0.25 cc. more is taken, crystals of barium carbonate now appeared after a few minutes. 1.5 cc. of the gas must contain, therefore, 1×10^{-7} gram of carbon dioxide.

From these duplicates, it becomes certain that 1.5 cc. out of 25 cc. of the chamber now contain 1×10^{-7} gram of carbon dioxide. Therefore the total amount of carbon dioxide produced by 10 mgms. of the tissue during ten minutes will be

$$1 \times 10^{-7} \text{ gram} \times \frac{25}{1.5} = 16.6 \times 10^{-7} \text{ gram of carbon dioxide}^{13}$$

5. For a rapid collection of air for a later analysis.

With this new apparatus one can also collect the air very rapidly and analyze it at leisure, thus enabling him to collect the air at successive short intervals of respiration by the same tissue or similar tissues at different stages of activity. For this purpose, a very simple special form of the gas pipette was devised. Figure

¹² This is the case when the analytic chamber has about 15–20 cc. It may take a longer time to produce a precipitate, when the chamber is much larger than this.

¹³ For correction for temperature and pressure, see footnote on p. 494.

2 will illustrate the exact shape of the tube. Instead of tube *B*, this tube is connected to the arm of the stopcock 2 at *a* by means of rubber tubing,¹⁴ and the tube is connected to the mercury burette *G* at *b*.

With this arrangement, we should repeat the experiment exactly the same way as above, except that when we drive the air from the chamber to this tube, we should drive it so far as to push a few cubic centimeters of mercury also from the chamber, so that the mercury will remain in the U-tube *U*, thus automatically sealing



FIG. 2. A special gas pipette $\frac{1}{2}$ actual size.

the tube. By clamping the rubber connection at *b*, this tube is removed,¹⁵ and another pipette is connected, and the experiment is repeated with the same tissue or another tissue as the case may be. By this method, one can collect twenty or thirty samples of the gas a day with a single apparatus.

¹⁴ Use of rubber tubing is harmless, provided the mercury burette *G* is kept above the level of stopcock 2.

¹⁵ Since this pipette has a capacity of 10–20 cc., and only about 10–15 cc. of air are introduced, there will usually be 4–5 cc. of mercury left at the lower end of the tube, thus sealing it automatically at both ends. It is obvious that one should keep the tube vertical, in order to keep it air-tight.

When we are ready to analyze the gas from these tubes, all we have to do is to connect the tube to the usual place (at 2) and raise the mercury burette *G* in such a way that the mercury in the U-tube is now driven up to fill all the capillary tubes between the chamber and the pipette, thus forcing all the atmospheric air out of the tube, and then stopcock 2 is turned so as to cut all connections. The air in the tube is then examined according to the method described before.¹⁶

¹⁶ One disadvantage of this new apparatus is that we must take into consideration temperature and pressure variation, which was entirely unnecessary for the previous apparatus. If the respiration and analysis were done at different temperature and pressure, the ratio between the minimum volume which gives the first precipitate and original volume of the chamber will not be rigid. In that case, the minimum volume should be translated to the volume at the temperature and pressure at the time of respiration. Such correction, however, will not be necessary if the analysis is done immediately after the respiration, during which the variation in temperature and pressure will not affect the result beyond the experimental error, as is shown in the following calculation:

Let us suppose that 10 mgms. of tissue respire for ten minutes at 18° at 760 mm. of pressure in 25 cc. of the chamber, and suppose 1.5 cc. of the same air at 22° , at 730 mm. of pressure (making a liberal estimate of the change in temperature and pressure) gave the first precipitate; then we will obtain the following results:

a. Without any correction, we get 1×10^{-7} gram $\times \frac{25}{1.5} = 16.6 \times 10^{-7}$ gram.

b. With the correction, 1×10^{-7} gm. $\times \frac{25}{1.5 \times \frac{(270+18) \times 730}{(270+22) \times 760}} = 17.6 \times 10^{-7}$ gm.

This is a little over 5 per cent error, which will be the maximum, and almost an impossible variation for ordinary weather in the laboratory for a short interval of time. Besides, we are dealing with a very small sample of moist tissue, the weight of which may easily vary within 5 per cent.

ON THE RATE OF ABSORPTION OF CHOLESTEROL FROM THE DIGESTIVE TRACT OF RABBITS.¹

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(Received for publication, November 24, 1913.)

Our knowledge of the physiology of cholesterol, particularly of its absorption from food, has only in recent years made notable progress. The fact that it is, in truth, absorbed from the food, according to the uniform evidence of several investigators, may now be considered established.

The following studies were undertaken in order to determine, as accurately as possible, the rate of absorption in normal rabbits, as a basis for further investigations of the alterations of this rate in those pathological conditions, notably lipemia, in which the cholesterol metabolism is known to be greatly disturbed.

As early as 1867, Tolmatschek³ sought to prove the absorption of cholesterol from the food by estimating the intake and output of cholesterol in the breast-fed child. In 1890, Thomas,⁴ working on dogs with experimental biliary fistula, found no increase of cholesterol in the bile with a diet rich in cholesterol. Jankau,⁵ two years later, found no increase in the feces of rabbits and dogs after a single feeding of cholesterol; but, six hours after feeding, he found also no increase in cholesterol in blood and bile and liver-substance. With this contradictory evidence he was forced to leave the question still undecided.

In 1906, the first work of real significance appeared. Pribram⁶ fed rabbits through a stomach-tube on three successive days with the palmitic

¹ These studies were made under the direction of Prof. P. Morawitz, to whom I wish here to express my gratitude, not only for valuable advice and assistance, but also for the opportunity to carry on the investigation.

² John Harvard Fellow, Harvard Medical School.

³ Tolmatschek: *Hoppe-Seyler's Med.-chem. Untersuchungen*, 1867, p. 272; cited from *Oppenheimer's Handbuch der Biochemie*, 1908, Vol. IV, No. 1, p. 485.

⁴ Thomas: *Inaug. Diss.*, Strassburg, 1890.

⁵ Jankau: *Arch. f. exp. Path. u. Pharm.*, xxix, p. 237, 1892.

⁶ Pribram: *Biochem. Zeitschr.*, i, p. 413, 1906.

and oleic acid esters of cholesterol and with cholesterol itself, in amounts of 1 gram on each day. One or two days later, the animal was killed and the blood and organs were analyzed for cholesterol by saponification, ether extraction and weighing. He found with feeding of esters and pure substance alike, an increase in the blood and no definite reliable results from the tissue estimations. He also showed that the serum of an animal fed with cholesterol would prevent or delay in small doses the haemolytic action of saponin on normal red blood corpuscles:

Morgenroth and Reicher⁷ reported in the next year, the following experiments with a series of rabbits. Rabbit A was fed 4 grams of cholesterol in 15 cc. of olive oil daily, rabbit B, the same amount of pure olive oil, rabbit C, nothing beyond the ordinary diet; all three were then injected alike with lecithide. After five days, the haemoglobin estimations were respectively 58, 20, and 30 per cent; and the cholesterol percentage in the blood, respectively 0.48, 0.03, and 0.026. The three sera showed corresponding influences on saponin haemolysis *in vitro*. These results confirm Pribram.

Goodman,⁸ in the same year, fed two sets of dogs respectively, white of egg and calves' brains, and found no increase in the bile with the diet richer in cholesterol.

In 1908, Kusumoto⁹ fed dogs with ordinary diet with and without the addition of cholesterol, and estimated the cholesterol of the feces, finding that an average of 30 per cent of the amount ingested failed of excretion through the intestines.

In the same year Dorée and Gardner,¹⁰ in a convincing series of experiments, supported the above cited evidence favoring absorption of cholesterol from the food. In the feces of rabbits fed in the course of several days with 2 grams of cholesterol, preceded and followed by three days of feeding with a cholesterol-free diet, they found that at least 25 per cent, and often more, of the ingested cholesterol failed to appear in the feces. A rabbit receiving only the cholesterol-free diet excreted no cholesterol. In blood-estimations they employed the gravimetric method. Rabbits fed twenty days with cholesterol-free diet showed only a trace of the substance in the blood, whereas 0.0415 per cent cholesterol appeared in the blood of rabbits fed for ten days on the same diet with the addition of a total of 2.25 grams of cholesterol. With dogs they found some increase in the blood with foods rich in cholesterol. Fraser and Gardner¹¹ reported inhibition of saponin haemolysis by the serum of rabbits fed (1) with cholesterol, (2) with the cholesterol esters, (3) with mixed diet, as compared in each case with the serum of rabbits fed on cholesterol-free bran. The same authors,¹² a year later, employing a modification of the new Windaus digitonin method of cholesterol estima-

⁷ Morgenroth and Reicher: *Berl. klin. Wochenschr.*, xxxviii, p. 1200, 1907.

⁸ Goodman: *Hofmeister's Beiträge*, ix, p. 91, 1907; cited from Dorée and Gardner: *loc. cit.*

⁹ Kusumoto: *Biochem. Zeitschr.*, xiv, p. 411, 1908.

¹⁰ Dorée and Gardner: *Proc. of the Royal Society*, lxxxi, p. 109, 1908.

¹¹ Fraser and Gardner: *Proc. of the Royal Society*, lxxxi, p. 230, 1909.

¹² *Ibid.*, lxxxii, p. 559, 1910.

tion, repeated the work of Dorée and Gardner and reported similar results. Studies carried out in 1912 by Ellis and Gardner,¹³ in the same laboratory, led them to conclude from evidence of the same nature, that the cholesterol content of the blood is dependent on the "sterol-content"—i. e., phytosterol and cholesterol—of the diet. An increase in the blood during starvation they attributed to the freeing of cholesterol by the destruction of the cholesterol-rich tissues of the animal.

Klein,¹⁴ two years earlier, as reported by Magnus-Levy, found increase of absorption with increase of dose of cholesterol, as measured by the output in the feces of dogs. He found no difference in absorption between pure cholesterol and its esters.

Grigaut and L'Huillier,¹⁵ in 1912, studied, with the former's colorimetric method, the curve of cholesterol content in the blood of dogs fed daily with 1 or 2 grams of cholesterol; and compared this curve with the curve of cholesterol present in the feces during the period of experimentation. His curves show a marked rise with the first feeding of cholesterol and a maintenance of the "hypercholestérinémie" throughout the feeding period, with a prompt fall coincident with the return to normal diet.

Anitschkow¹⁶ found, after prolonged feeding of cholesterol, pathological changes in various tissues of rabbits, particularly the walls of the aorta, representing an increased body-content of cholesterol. Others report similar observations.

Rouzaud and Cabanis,¹⁷ using the Grigaut method, found an increase in the blood of only one out of eleven healthy young people, four to five hours after the ingestion of a meal consisting of thin soup, bread, meat, green peas, two eggs and wine. The other ten subjects showed no change. Their work seems not to have been satisfactorily controlled.

For the purpose of this research, the sole conclusion to be drawn from these investigations is that, after the feeding of cholesterol and its esters in relatively large doses, there is an undoubted increase in the blood as compared to the average figures with any one method of estimation. They give no hint as to the rapidity of absorption into the blood or disappearance from it.

METHOD.

For the present investigation, rabbits were fed alike on the ordinary mixed diet of oats, hay or grass, and bread, and received daily as much as they would eat. They were thus under approximately normal conditions of metabolism.

¹³ Ellis and Gardner: *Proc. of the Royal Society*, lxxxv, p. 385, 1912.

¹⁴ Klein (Magnus-Levy): *Biochem. Zeitschr.*, xxix, p. 465, 1910.

¹⁵ Grigaut and L'Huillier: *Compt. rend. soc. biol.*, lxxiii, p. 304, 1912.

¹⁶ Anitschkow: *Deutsch. med. Wochenschr.*, 1913, p. 741.

¹⁷ Rouzaud and Cabanis: *Compt. rend. soc. biol.*, lxxiv, p. 469, 1913.

About 6 cc. of blood were withdrawn for estimation and immediately thereafter 10 cc. of a 3 per cent solution of cholesterol in olive oil (Merck), representing a dose of 0.3 gram of cholesterol, was introduced into the stomach by a tube. The control animals, with the exception of one (XVII), received the same amount of a solution of pure olive oil.

The blood for the further estimations was withdrawn at intervals indicated in the tables to follow, in each instance about 6 cc. being taken. The degree of the consequent anemia was observed by haemoglobin determinations after Haldane. All bleeding was done by the Zahn method¹⁸ with a suction-glass from the veins of the ear; and the blood was treated with sodium oxalate to prevent coagulation.

The Autenrieth-Funk¹⁹ colorimetric method of cholesterol estimation, with chloroform extraction, was employed, and, owing to the small amount of blood required by this method, it was possible, in most instances, to make two independent extractions and determinations with each sample of blood. The results of the two determinations, in most cases, agreed closely, as the tables indicate. On account of the small percentage of cholesterol that the bloods yielded, it was found necessary to modify the technique, as the authors suggest in the original description, to the extent of extracting with 55 cc. instead of 100 cc., and, after the ordinary incidental evaporation, making up the test solution to 50 cc. Repeated further extractions with no addition to the yield of cholesterol showed that, with small percentages at least, the less extended extraction is as effective as a more thorough procedure.

RESULTS OF EXPERIMENTS.

The figures for the cholesterol content from the blood of twenty normal rabbits, as given in the seventeen tables below, together with those from three rabbits not there listed, range from a maximum of 0.1230 to a minimum of 0.0795 per cent with an average of 0.1020 per cent. This average is distinctly higher than that which Abderhalden²⁰ reported in 1898, determined by the gravimetric method from the mixed blood of twelve healthy rabbits. His figure for the whole blood is 0.0611 per cent.

¹⁸ Zahn: *Münch. med. Wochenschr.*, 1912, No. 16, p. 861.

¹⁹ Autenrieth and Funk: *Münch. med. Wochenschr.*, 1913, No. 23, p. 1243.

²⁰ Abderhalden: *Zeitschr. f. physiol. Chem.*, xxv, p. 65, 1898.

The records of the seventeen experiments and controls follow:

Experimental series. Fed with cholesterol.

Rabbit I. 1550 grams. 10 cc. 3 per cent cholesterol oil by mouth.

INTERVAL AFTER ADMINISTERING CHOLESTEROL	Hgb.	CHOLESTEROL IN BLOOD				
		Absolute Percentage			Increase	Decrease
		a	b	Average		
	per cent				per cent	per cent
Normal.....	74	0.1200	0.1195	0.1198		
6 hours.....		0.1185	0.1195	0.1190		0.67
3 days.....	60	0.1515	0.1490	0.1503	25.46	
19 days.....	73	0.1090	0.1050	0.1070		10.68

Rabbit II. 1650 grams. 10 cc. 3 per cent cholesterol oil by mouth.

Normal.....	60	0.1230		0.1230		
24 hours.....		0.1550	0.1580	0.1565	27.23	
5 days.....		0.1515	0.1535	0.1525	23.99	
19 days.....	60	0.1400		0.1400	13.82	

Rabbit III. 1650 grams. 10 cc. 3 per cent cholesterol oil by mouth.

Normal.....	75	0.1010	0.1020	0.1015		
6 hours.....		0.1050	0.1040	0.1045	2.95	
3 days.....	68	0.1074	0.1050	0.1062	4.63	
5 days.....	60	0.0950	0.0945	0.0948		6.60

Rabbit IV. 1520 grams. 10 cc. 3 per cent cholesterol oil by mouth.

Normal.....	80	0.0920	0.0920	0.0920		
24 hours.....		0.1190	0.1175	0.1183	28.58	
4 days.....	76	0.1050		0.1050	14.13	
6 days.....	70	0.1065	0.1050	0.1058	15.00	
18 days.....	78	0.1015	0.1040	0.1028	11.74	

Rabbit V. 2340 grams. 10 cc. 3 per cent cholesterol oil by mouth.

Normal.....	75	0.0900	0.0860	0.0880		
8 hours.....		0.0960	0.0965	0.0963	9.43	
2 days.....		0.0965	0.0940	0.0953	8.29	

Rabbit VI. 2570 grams. 10 cc. 3 per cent cholesterol oil by mouth.

Normal.....	70	0.1120		0.1120		
12 hours.....		0.1190	0.1165	0.1178	5.18	
3 days.....		0.1170	0.1050	0.1110		0.89

*Experimental series. Fed with cholesterol—Continued.**Rabbit VII.* 1930 grams. 10 cc. 3 per cent cholesterol oil by mouth.

INTERVAL AFTER ADMINISTERING CHOLESTEROL	Hgb.	CHOLESTEROL IN BLOOD				
		Absolute Percentage			Increase	Decrease
		a	b	Average		
	per cent				per cent	per cent
Normal.....	70	0.1090	0.1105	0.1098		
12 hours.....		0.1190	0.1220	0.1205	9.74	
3 days.....		0.1685	0.1715	0.1700	54.83	
6 days.....	64	0.1720	0.1780	0.1750	59.38	

Rabbit VIII. 1700 grams. 10 cc. 3 per cent cholesterol oil by mouth.

Normal.....	76	0.1070	0.1060	0.1065		
24 hours.....		0.1150	0.1170	0.1160	8.92	
3 days.....	68	0.1055	0.1000	0.1028		3.47

Rabbit IX. 1750 grams. 10 cc. 3 per cent cholesterol oil by mouth.

Normal.....	92	0.0900	0.0910	0.0905		
6 hours.....		0.0795	0.0820	0.0808		10.72
2 days.....	75	0.1130		0.1130	24.86	

Rabbit X. 1700 grams. 10 cc. 3 per cent cholesterol oil by mouth.

Normal.....	96	0.0810	0.0820	0.0815		
6 hours.....		0.0730	0.0825	0.0778		4.54
3 days.....	82	0.0955	0.0950	0.0953	16.93	

Rabbit XI. 1610 grams. 10 cc. 3 per cent cholesterol oil by mouth.

Normal.....	74	0.0910	0.0910	0.0910		
8 hours.....		0.0790	0.0900	0.0845		7.14

Rabbit XII. 1570 grams. 10 cc. 3 per cent cholesterol oil by mouth.

Normal.....	70	0.0855	0.0880	0.0868		
8 hours.....		0.0680		0.0680		20.65

*Control series.**Rabbit XIII.* 1450 grams. Control. 10 cc. olive oil by mouth.

INTERVAL AFTER ADMINISTERING OLIVE OIL	Hgb.	CHOLESTEROL IN BLOOD				
		Absolute Percentage			Increase	Decrease
		a	b	Average		
	<i>per cent</i>				<i>per cent</i>	<i>per cent</i>
Normal.....	78	0.0865	0.0870	0.0868		
6 hours.....		0.1100	0.1095	0.1098	26.49	
3 days.....	60	0.0972		0.0972	11.98	

Rabbit XIV. 1480 grams. Control. 10 cc. olive oil by mouth.

Normal.....	82	0.1100	0.1095	0.1098		
6 hours.....		0.1185	0.1170	0.1178	7.29	
3 days.....	68	0.1110	0.1140	0.1125	2.46	
5 days.....	62	0.1150		0.1150	4.73	

Rabbit XV. 1700 grams. Control. 10 cc. olive oil by mouth.

Normal.....	72	0.0770	0.0820	0.0795		
12 hours.....		0.0760	0.0780	0.0770		3.14
3 days.....		0.0815	0.0790	0.0803	1.00	

Rabbit XVI. 1900 grams. Control. 10 cc. olive oil by mouth.

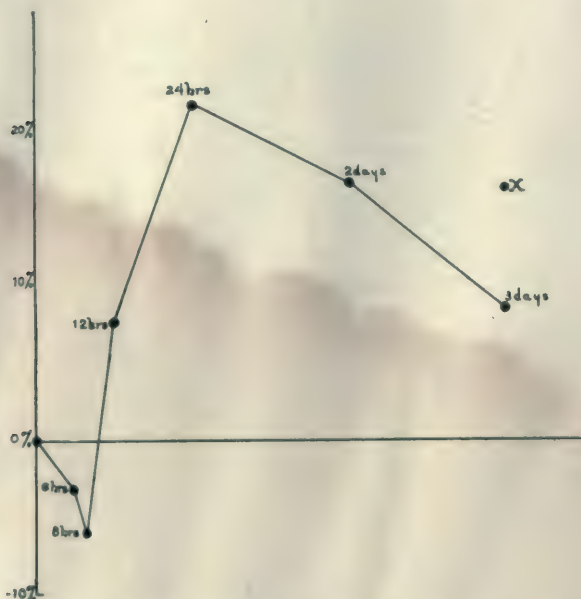
Normal.....	70	0.0990	0.1020	0.1005		
24 hours.....		0.1015	0.1045	0.1030	2.48	
4 days.....	70	0.1000	0.0955	0.0978		2.68

Rabbit XVII. 1920 grams. Control. Received nothing.

Normal.....	76	0.1130	0.1145	0.1138		
6 hours.....		0.1070	0.1095	0.1083		4.83

From the above figures the appended curve was constructed, showing the average blood-content at the intervals indicated in the twelve animals that received the usual dose of cholesterol. It must be noted, that, although the points form a not irregular line, the average figure for any one interval is, in most cases, drawn from widely differing individual figures. Thus at six hours, the average indicated on the curve, -3.24 per cent, is a product of results varying from -10.72 per cent to +2.95 per cent. In reckoning the

average figure for three days, it was necessary to omit the record of Rabbit VII, which shows at that interval an increase twice as great as the next greatest in the series and six times as great as the average; this discrepancy is not to be accounted for. The point X shows the deflection of the curve if the cholesterol content of the blood from Rabbit VII is allowed to enter as an element in the average.



Composite curve from seventeen rabbits, showing the average variation in cholesterol content of the blood after the feeding of 0.3 gram of cholesterol.

The great differences in the individual reactions both in the experimental animals and in the controls, do not appear to be dependent on the body weight. Although Rabbits V and VI, the heaviest in the series, are among those showing the least effect from the procedure, yet Rabbit III, about 1000 grams lighter, shows even less effect, and Rabbit VII, 300 grams heavier than Rabbit III, shows the greatest effect of all. That the repeated withdrawal of blood is alone sufficient to cause great variations from the normal content is suggested by Mauriac.²¹ This is quite

²¹ Mauriac: *Compt. rend. soc. biol.*, lxxiii, p. 675, 1912.

possible, but an analysis of the figures shows that the control animals show less average variation from the normal than the experimental animals. There is one notable exception; a single control, Rabbit XIII, fed with pure olive oil, at the end of six hours showed an increase of 26.49 per cent whereas among the animals fed with cholesterol it will be noted that in only one instance (III) was there an increase so soon after feeding, and in that case an increase of only 2.95 per cent. The reason for this exceptional occurrence in Rabbit XIII is unexplained. This, however, can hardly vitiate the conclusion that, in general, the curve established from the estimations on the blood of the cholesterol-fed animals is based on actual absorption of the cholesterol placed in the digestive tract and not on properties of the technique employed.

To turn to the figures from the cholesterol-fed animals from which the curve was constructed, we find that several of the animals, as mentioned above (III, V, VI), show less, or little more, increase of cholesterol in the blood after the administration of cholesterol than do the animals that received no cholesterol. The majority however, tend to correspond with the curve—to show an initial fall (due, perhaps, to the recent hæmorrhage with dilution of the diminished blood-volume from tissue-fluids) lasting for six to eight hours, followed by a rise reaching its maximum at the end of about twenty-four hours, and a more gradual fall through the next two or more days.

The small number of experiments performed does not permit more extended generalization; one must expect that any individual rabbit may depart widely from the tendency that the curve expresses.²²

CONCLUSIONS.

It is possible by giving rabbits small doses of cholesterol by mouth to demonstrate in the majority of instances, an increase of this substance in the blood in the course of a few hours.

²² Three experiments, with subcutaneous injection of cholesterol oil in 10 cc. amounts suggest that the absorption from the subcutaneous tissues is much slower. The maximum amount in the blood appeared to be reached between the third and sixth days or even later. These animals were not controlled and the other conditions of the experiments make the results worth recording only as being suggestive.

GLYOXALASE. PART IV.

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The object of the following paper is to record some new experiments dealing with enzymes of the glyoxalase type and with the inhibitory action of the pancreas upon these enzymes. It may be recalled that we have recorded the presence of glyoxalases in a variety of tissues from various animal species.¹ The tissues examined with positive results included liver, kidney, thyroid, spleen, heart muscle, skeletal muscle, tongue, lung, brain, blood cells and gastric mucosa. Negative results were obtained with saliva, urine, bile and blood serum. On the other hand, pancreatic tissue and juice were found not only to be free from glyoxalase but to contain a thermolabile substance, not improbably an enzyme, which exerts an intense inhibitory action upon glyoxalase derived from other sources. The inhibitory substance, named for convenience antiglyoxalase, is not identical with trypsin, lipase or diastase.

The inhibitory action of the pancreas upon glyoxalase appeared so definite a phenomenon and had so suggestive a relation to the function of the pancreas in carbohydrate and lactic acid metabolism, that we considered it desirable to examine other glands of the body, particularly those which in recent years have been brought into relation with sugar metabolism, in order to learn if antiglyoxalase was peculiar to the pancreas.

We have been unable to obtain any evidence of the presence of antiglyoxalase in the thymus, thyroid, suprarenal, pituitary, or salivary glands, or testicle, but on the contrary we have determined the presence of glyoxalase in all of these organs. The amount of glyoxalase in the salivary glands is very small. In the case of the abdominal lymph glands of the dog, we have observed a con-

¹ This *Journal*, xv, p. 463, 1913.

stant absence of glyoxalase, but efforts to obtain evidence of the presence of antiglyoxalase have given doubtful or negative results. It is certain, however, that if antiglyoxalase be present in the lymph glands, its amount is utterly insignificant when compared with the pancreas. It is perhaps conceivable that the antiglyoxalase of the pancreas reaches distant parts of the body by way of the lymphatic system, but of this we have no precise evidence. Moreover, we are inclined to believe that, notwithstanding the fact that antiglyoxalase is present in the external pancreatic secretion, it is likely that antiglyoxalase is largely contained in an internal secretion. The reasons for this belief are as follows: In the first place we are under the impression that the concentration of antiglyoxalase in pancreatic juice is relatively small compared with that in the tissues. Secondly, antiglyoxalase appears to be non-dialyzable and does not pass through animal membranes and hence would probably not undergo ready absorption from the intestine. In the light of our present results, it appears that the production of antiglyoxalase is a specific function of the pancreas.

We wish to record at this point that in a private communication from Prof. F. G. Hopkins we learn that in some experiments made several years ago, he found that lactic acid production in muscle was markedly inhibited by the action of pancreas extract. We understand that these experiments are being amplified and will be published shortly.

In a recent paper Neuberg has taken exception to the name glyoxalase. He writes:²

Das Enzym, das die Umwandlung von Methylglyoxal in Milchsäure bewirkt, ähnelt nach meinen Ausführungen in seinen Eigenschaften und seiner Wirkungsweise der bekannten Aldehydmutase. Durchgreifende Unterschiede von dieser sind nicht offenbar geworden. Es liegt daher bislang keine Veranlassung vor, das Ferment als Vertreter einer neuen Gruppe zu betrachten, wie es Dakin tut. Besonders ist aber der von Dakin gewählte Name "Glyoxalase" höchst unglücklich, da gerade das Glyoxal bisher nicht nachweisbar beeinflusst wird. Zweckmässiger erscheint daher mein Vorschlag (l. c.), das Enzym den Aldehydasen anzureihen und es vorläufig Ketonaldehydmutase zu benennen, da diese Bezeichnung nichts präjudiziert.

² *Biochem. Zeitschr.*, lv, p. 502, 1913.

We believe that these objections are without weight for the following reasons. In the first place, contrary to Neuberg, we find that glyoxal is converted into glycollic acid by enzyme action. On perfusing a dog's liver with blood to which glyoxal had been added, we recovered almost 2 grams of pure calcium glycollate. Since ordinary commercial glyoxal is a mixture of highly polymerized substances, it is not surprising that it should be acted on less readily than some of the other glyoxals.

Secondly, we have found that the inhibitory action of pancreas extract upon glyoxalase furnishes us with an excellent method for the differentiation of glyoxalase from other enzymes. Parnas' aldehydemutase is scarcely affected by pancreas extract under conditions which completely inhibit glyoxalase, so that we believe that the two enzymes can have nothing in common. Incidentally it may be noted that the distribution of the two enzymes in the body is quite different and the reactions with which they severally are concerned have only a superficial resemblance. We therefore propose to retain the name "glyoxalase" for the enzymes which we have shown to effect the conversion of various glyoxals into the corresponding hydroxy-acids:



Thus far we have made use of glyoxal itself, methyl glyoxal, isobutyl glyoxal, phenyl glyoxal and benzyl glyoxal, and in every case we have obtained the corresponding hydroxy-acid by the action of glyoxalase. Finally we wish to mention that we have been able to demonstrate the formation of amino- as well as hydroxy-acids from corresponding glyoxals when perfused through the liver. We are also making experiments in which the formation of hydroxy-acid is suppressed by addition of pancreas extract to the blood used for perfusion. A description of the synthesis of the hitherto unknown isobutyl and benzyl glyoxals, corresponding to leucine and phenylalanine, together with a study of the formation of amino- and hydroxy-acids from them, will be published shortly.

EXPERIMENTAL.

I. Glyoxalase in certain glands.

The method employed was identical with that already described in a previous paper,³ except that in these experiments instead of using extracts the tissues themselves after washing free from blood were added to the digestion mixtures. Phenyl glyoxal was used as substrate and in all positive experiments the mandelic acid produced was isolated in crystalline form.

From blank experiments in which the enzyme was destroyed by preliminary heating, no mandelic acid could be obtained. The following results are typical.

ANIMAL	TISSUE		ACIDITY	ROTATION OF MANDELIC ACID	PRESENCE OF GLYOX- ALASE
			cc.	deg.	
Calf.....	Thymus	(10 gms.)	2.2	-0.53	+
Horse.....	Suprarenal	(20 gms.)	10.0	-2.56	+
	Pituitary	(3 gms.)	1.7	-0.27	+
Ox.....	Pituitary	(10 gms.)	5.4	-1.25	+
	Testicle	(10 gms.)	5.8	-1.75	+
Dog.....	Abdom. lymph glands	(5 gms.)	0.8	0	-
	Abdom. lymph glands	(6 gms.)	1.2	+0.05	-
	Salivary glands	(10 gms.)	2.2	-0.22	+

We owe the experiments on the suprarenal and pituitary glands of the horse to the kindness of Dr. C. Ten Broeck of Harvard, and we wish to express our thanks also to Dr. F. Fenger of the research laboratories of Armour and Company, for making the experiments with ox pituitary.

II. Examination of abdominal lymph glands of the dog for antiglyoxalase.

Many experiments were made in which finely chopped lymph glands⁴ were added to 20 per cent skeletal muscle extract. After

³ This *Journal*, xv, p. 466, 1913.

⁴ Glands from near the pancreas were purposely rejected.

incubating this mixture for several hours in the presence of chalk, phenyl glyoxal was added and incubation continued for about eighteen hours. As control an equal amount of 20 per cent extract was incubated in the presence of chalk, phenyl glyoxal being added at the same time as to the lymph gland experiment. Only a few typical experiments are here reported.

a. 50 cc. 20 per cent skeletal muscle extract were incubated for three hours with 9 gms. minced lymph glands and 5 cc. of a chalk suspension. Then 0.2 gm. phenyl glyoxal was added and incubation continued for twenty hours.

In the control experiment 50 cc. of the same muscle extract were incubated in the presence of chalk for three hours, after which 0.2 gm. phenyl glyoxal was added and incubation continued for twenty hours.

Lymph gland experiment. Rotation of mandelic acid: -0.82° ; Acidity, 3.8 cc.

Control. Rotation of mandelic acid: -1.35° ; Acidity, 3.8 cc.

b. As in (*a*), 14 gms. lymph glands being used.

Lymph gland experiment. Rotation of mandelic acid: -0.37° ; Acidity, 3.2 cc.

Control. Rotation of mandelic acid: -0.22° ; Acidity, 2.0 cc.

c. As in (*a*) 5 gms. lymph glands taken.

Lymph gland experiment. Rotation of mandelic acid: 0° ; Acidity, 1.8 cc.

Control. Rotation of mandelic acid: -0.32° ; Acidity, 1.8 cc.

d. As in (*a*) 3.5 gms. lymph glands added.

Lymph gland experiment. Rotation of mandelic acid: -0.18° ; Acidity, 2.2 cc.

Control. Rotation of mandelic acid: -0.17° ; Acidity, 2.2 cc.

The results of these experiments show that under the observed conditions the presence of antiglyoxalase in lymph glands cannot be definitely asserted.

III. Dialysis experiments with antiglyoxalase.

Ten grams of fresh dog's pancreas were ground up with sand mixed with 50 cc. of water and dialyzed for twenty hours in a condome made from the caecum of the sheep. This was chosen as an appropriate membrane to employ in this experiment. The contents of the condome and the dialysate were then tested for antiglyoxalase by adding them each to 50 cc. of 20 per cent dog's skeletal muscle extract and incubating for three hours before adding phenyl glyoxal, at the same time carrying out a control

experiment with the muscle extract. The following results were obtained:

Muscle extract. Rotation of mandelic acid: -0.9° ; Acidity, 4.4. cc.

Muscle extract + contents of dialyzer. Rotation of mandelic acid: $+0.1^{\circ}$; Acidity, 0.5 cc.

Muscle extract + dialysate. Rotation of mandelic acid: -0.85° ; Acidity, 4.6 cc.

It will be noted that there is no inhibition in the third experiment, whereas in the second the enzyme has been completely paralyzed showing that no antiglyoxalase has passed through the membrane.

IV. Formation of glycollic acid from glyoxal in the liver.

The technique of perfusion was similar to that already reported⁵ in connection with other experiments made in this laboratory. The dog (4.8 kgm.) was starved for twenty-four hours previous to the operation. The liver (170 grams) was perfused with a mixture containing 170 cc. of the animal's own blood, 500 cc. of fresh blood from another dog and 200 cc. of saline. During the first ten minutes of the perfusion solutions of about 5 grams glyoxal in 100 cc. water and of 5 grams sodium bicarbonate in 200 cc. water were added in small portions to the perfusion fluid. At the end of the perfusion, which lasted an hour, the liver was washed out with 200 cc. of saline. The perfusion fluid, after removal of proteins according to Schenck's method, when tested with *p*-nitrophenylhydrazine, gave a precipitate of the characteristic dinitrophenylhydrazone of glyoxal, showing that there was still unchanged glyoxal present.

An acetone determination of an aliquot part of the filtrate showed that only 29 mgm. of acetoacetic acid had been formed.

The mercury was removed from the clear filtrate of the perfusion fluid by means of hydrogen sulphide and the still acid liquid was then evaporated to dryness *in vacuo*. The residue was washed with alcohol, the alcohol extract evaporated, taken up in water and after the addition of ammonium sulphate and phosphoric acid, extracted with ether in a continuous extractor. The ether

⁵ This *Journal*, ix, p. 146, 1911.

extract, after treatment with calcium carbonate gave a salt crystallizing in the characteristic form of calcium glycollate.

Almost 2 grams of crystallized calcium glycollate were obtained.

Analysis of air-dried salt: 0.1176 gm. lost 0.0260 gm. H_2O at 140° and gave 0.0269 gm. CaO .

	Found:	Calculated for $CaC_2H_5O_6 \cdot 3H_2O$:
H_2O	22.1	22.1 per cent.
Ca.....	16.4	16.4 per cent.

The remainder of the calcium salt was decomposed with oxalic acid, the filtrate evaporated and taken up in ether. The residue from the clear ether solution crystallized at once on seeding with a crystal of glycollic acid, and the crystals so obtained, dried on porous plate, melted at $76-78^\circ$.

V. *The differentiation of glyoxalase from aldehydemutase by means of the action of pancreas extract.*

As is known, an extract of pancreas inhibits the action of glyoxalase. The following experiment was made to determine whether pancreatic extract exerted a similar effect on aldehydemutase, an enzyme described by Battelli and Stern⁶ and also by Parnas,⁷ whose method of investigation was substantially followed.

The enzyme extract was made by stirring water with an equal weight of minced ox liver and straining through muslin. 250 cc. of this extract were then placed in each of four flasks. To the contents of flasks (1) and (2) were added 2.1 grams of sodium bicarbonate and 2 cc. of isovaleric aldehyde. The extract in flask (3) was digested forty minutes with 1 gram pancreatin before adding 2.1 grams sodium bicarbonate and 2 cc. isovaleric aldehyde. Flask (4) was heated on the water bath for fifteen minutes and then similar amounts of sodium bicarbonate and isovaleric aldehyde were added.

All the mixtures were incubated at 37° for five and one-half hours after addition of the aldehyde. The reaction was then checked by adding 10 cc. of phosphoric acid, and the mixtures were immediately distilled in steam, 500 cc. of distillate being collected

⁶ *Biochem. Zeitschr.*, xxviii, p. 147, 1910; xxix, p. 130, 1910.

⁷ *Ibid.*, xxviii, p. 274, 1910.

in each case. The distillates were then titrated, the results being as follows:

- (1) Acidity = 6.7 cc.
- (2) Acidity = 6.4 cc.
- (3) Acidity = 5.7 cc. Pancreas added.
- (4) Acidity = 2.7 cc. Blank experiment.

This experiment shows clearly that the action of the enzyme aldehydemutase is not abolished by pancreas extract, as is the case with glyoxalase.

A second experiment was made in order to compare directly the aldehydemutase and glyoxalase contained in the same extract both before and after treatment with pancreas extract. To this end an emulsion of dog's liver in five times its weight of water was taken. In two flasks (1) and (2) were placed 400 cc. of the emulsion and to flask (2) was added the dog's pancreas (16 grams) finely minced. In flask (3) 350 cc. of the liver emulsion were heated up on the water bath for fifteen minutes to act as a control in the aldehydemutase determinations.

After flasks (1) and (2) had been incubated at 37° for two hours, 50 cc. from each were measured off into flasks (4) and (5) respectively; to these were added 0.2 gram phenyl glyoxal and 5 cc. of a chalk suspension and a typical glyoxalase determination was carried out.

To each of flasks (1), (2) and (3) were added 3 grams of sodium bicarbonate and 3 cc. of isovaleric aldehyde. Incubation was continued for fifteen hours, when the reaction was checked by the addition of 15 cc. of phosphoric acid and the mixtures were immediately distilled in steam, 400 cc. being collected in each distillation. The distillates were titrated, giving figures indicative of the activity of aldehydemutase, while the determination of the mandelic acid produced in flasks (4) and (5) is a measure of the glyoxalase contents of the same mixtures.

Aldehydemutase: Flask 1. Pancreas absent. Acidity = 3.2 cc.

Flask 2. Pancreas present. Acidity = 3.0 cc.

Flask 3. Blank. Acidity = 1.6 cc.

Glyoxalase: Flask 4. Pancreas absent. Rotation of mandelic acid, -1.13°; Acidity, 2.0 cc.

Flask 5. Pancreas present. Rotation of mandelic acid, -0.12°; Acidity, 0.25 cc.

The glyoxalase determinations (4) and (5) were actually made on samples taken from flasks (1) and (2).

It is seen that the activity of aldehydemutase is not appreciably diminished by the action of pancreas extract, while the glyoxalase under the same treatment is practically completely inhibited. The two enzymes are undoubtedly distinct.

SUMMARY.

The presence of glyoxalase and the absence of antiglyoxalase has been determined in all the glands of the body we have examined, with the exception of the pancreas and abdominal lymphatic glands.

The lymphatic glands contain no glyoxalase and compared with the pancreas, the inhibitory action of extracts of lymph glands upon glyoxalase is trifling or non-existent.

The formation of antiglyoxalase, so far as can be at present determined, appears to be a specific function of the pancreas, and some reasons are adduced for suspecting that it acts mainly by way of an internal secretion.

Contrary to Neuberg's statement, we find that glyoxal may be converted into glycollic acid by enzyme action. Furthermore we find that Neuberg's suggested relation between glyoxalase and aldehydemutase is incorrect. We have shown that the enzymes are entirely distinct since, unlike glyoxalase, aldehydemutase is substantially unaffected by pancreas extract.



SOME NEGATIVE EXPERIMENTS ON THE INFLUENCE OF THE PANCREAS UPON ACETOACETIC ACID FORMATION IN THE LIVER.

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(*From the Herter Laboratory, New York.*)

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The incentive to make the following experiments to determine a possible influence of the pancreas upon acetoacetic acid formation in the liver arose from the following facts. First, the known acidosis with excretion of acetoacetic acid observed to follow extirpation of the pancreas. Second, the fact that we have found in the pancreas a mechanism for the regulation of the action of the enzyme glyoxalase, whose function, at least in part, is concerned with the formation of another acid produced in intermediary metabolism, namely, lactic acid. Third, the fact that it is much more difficult to evoke a considerable excretion of acetoacetic acid in the non-diabetic intact animal under normal conditions than it is to demonstrate its production in the excised liver on perfusion.

It seemed possible that the pancreas might furnish some enzyme or hormone the absence of which leads to acidosis in the diabetic animal. So far as we are aware, no experiments have hitherto been made to determine this point, although a number of workers have investigated the influence of the pancreas upon the capacity of various tissues to effect the oxidation of glucose. More recently Paderi¹ has found that the addition of pancreas extract to the fluid used for perfusing a glycogen-containing liver, was not followed by a diminished glucose production.

In our attempt to detect any influence that the pancreas may have on acetoacetic acid production we have perfused dogs' livers with blood containing added substances, known from Embden's

¹ *Arch. d. pharmacol. experim.*, xvi, p. 54, 1913.

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experiments to yield acetoacetic acid freely. Tyrosine and the sodium salts of butyric and homogentisic acids were the substances chosen. In some of the experiments we added fresh extract of pancreas to the blood used for perfusion, while in others we added skeletal muscle extract or heated pancreas extract to serve as a control. The tissue extracts were prepared by grinding up perfectly fresh tissue with sand and ten parts of distilled water. The suspension was then strained, a suitable quantity of salt added, then whipped with clotting blood and again strained before adding it to the blood used for perfusion. The methods of perfusion and analysis were those previously used by us.

Although the results of our experiments, taken literally, might be considered to show a slightly lessened acetoacetic acid production in those experiments in which pancreas extract was added to the blood, we believe that the results do not warrant any such interpretation, owing to wide individual variations in similar experiments.

We conclude that under the conditions of our experiments addition of pancreas extract to the blood has no marked effect upon acetoacetic acid formation in the liver from butyric acid, homogentisic acid or tyrosine.

SUBSTANCE ADDED	TISSUE EXTRACT ADDED TO BLOOD	WEIGHT OF LIVER	MILLIGRAMS ACETO-ACETIC ACID FORMED
		<i>grams</i>	
1. Butyric acid 2 gms.....	100 cc. pancreas.....	267	73
2. Butyric acid 2 gms.....	250 cc. pancreas.....	318	111
3. Butyric acid 2 gms.....	100 cc. heated pancreas	339	106
4. Butyric acid 2 gms.....	100 cc. muscle	261	112
5. Tyrosine 1 gm.....	100 cc. pancreas	223	89
6. Tyrosine 2 gms.....	200 cc. pancreas.....	267	74
7. Tyrosine 2 gms.....	200 cc. pancreas.....	169	114
8. Tyrosine 2 gms.....	200 cc. heated pancreas	281	126
9. Homogentisic acid 2 gms.	100 cc. pancreas.....	289	178
10. Homogentisic acid 2 gms.	200 cc. pancreas....	151	174
11. Homogentisic acid 2 gms.	100 cc. muscle	159	325

ON FAT ABSORPTION.

III. CHANGES IN FAT DURING ABSORPTION.

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The belief regarding fat absorption which is now almost universally accepted is that the fats are saponified in the intestine, absorbed in water-soluble form as soaps, and resynthesized into neutral fats during the passage through the absorbing cells. The more important evidence¹ in support of this belief is as follows:

1. Fatty substances such as glycerides, other readily saponifiable esters, fatty acids and soaps, lecithin, etc., which are soluble in water or can be changed by digestion into compounds soluble in water (or bile) at body temperature are readily absorbed. They appear in the chyle as triglycerides.

2. Fatty substances which cannot be changed into water-soluble form in the intestine under these conditions are not absorbed, *no matter in what form they are presented*. In this class fall difficultly saponifiable esters such as those of wool fat, also the petroleum hydrocarbons, etc.,—substances which are soluble in the ordinary fats and fat solvents, and in most cases form very good emulsions with water.

3. The abundant provision made for saponification and for the absorption of soaps in the intestine by the supply of large amounts of lipase and bile—whose chief function is now believed to be to aid in fat absorption.

4. The presence of soaps in the intestine in relatively large proportion, while only small amounts are present in the chyle.

5. While emulsified neutral fats are found on both sides of the absorbing cells, the particles of emulsified fat in the lacteals are

¹ The evidence is discussed in greater detail in the preceding paper of this series: this *Journal*, xv, p. 105, 1913.

very much finer than those in the intestine. They are of "dust-like fineness,"² apparently of the same order of magnitude as the "haemakonien" of Neisser,³ and are probably formed by the partial flocking of the newly resynthesized fat molecules under the influence of the electrolytes of the lymph stream.

The purpose of the complete breaking down of the fats in the intestine and their immediate resynthesis in the passage from the intestine is not clear in the light of our present knowledge. One reason which has been suggested⁴ is that the process is a protective one for the purpose of excluding undesirable fatty substances, such as wool-fat and the petroleum hydrocarbons, which differ from ordinary food fats mainly in that they cannot be changed into water-soluble substances in the intestine, and which but for this mechanism would be absorbed with the fats. But this reason is obviously not sufficient to explain the changes, since these substances rarely occur in the food. A comparison of the absorption of fats with that of proteins and carbohydrates suggests another reason. It is now believed that during digestion all (organic) food-stuffs alike are broken down into their component "Bausteine" in the intestine, the object being to provide material in a sufficiently elementary form for use in building up the characteristic body tissue. Proteins are broken down to amino-acids, carbohydrates to monosaccharides and fats to fatty acids and glycerins. Protein and carbohydrate "Bausteine" pass directly into the blood stream and are not rebuilt into body protein and carbohydrate complexes (accepting the findings of Folin and Denis) until they reach the tissues and organs. In their passage from the intestine to the system they pass through the liver. Fats are unique in that they are rebuilt *before they leave the intestinal wall*, and entering the blood stream by way of the thoracic duct, avoid the liver, which appears to take part in fat metabolism only after the fats have passed to the tissues. The protein complexes rebuilt by the tissues from the protein building stones are different from the proteins ingested and are characteristic of the species, and of the tissue. Since the fats are rebuilt during their passage through the intestinal wall, it is logical to expect a change in their chemi-

² Munk: *Virchow's Archiv*, cxxiii, p. 239, 1891.

³ Neisser and Bräuning: *Zeitschr. f. exp. Path. u. Ther.*, iv, p. 747, 1907.

⁴ Bloor: *loc. cit.*

cal structure during resynthesis analogous to that undergone by the proteins in the tissues. Another, and perhaps the main reason, then, for the phenomena of fat absorption may be looked for in changes which the fats undergo during absorption. What changes may be expected in the fats during absorption? The chemical structure of the fats is not definitely known, but it is believed that many fats contain in addition to simple triglycerides a considerable proportion of mixed triglycerides. The presence of mixed triglycerides is significant because many lecithins and similar substances contain a mixed glyceride residue; and since these lipoids are more closely identified with tissue structure than the fats, it may be assumed that the mixed glycerides are of special importance in those phases of fat metabolism which have to do with tissue repair. That mixed glycerides are also potentially optically active may also be considered significant in view of the possible importance of molecular structure in the utilization of other foodstuffs.⁵

We may look for two kinds of change in the fats during their passage through the intestinal wall: (a) a physical change consisting in a rearrangement of the quantities of the different glycerides—more or less of the liquid fats or of the solid fats—resulting in a mixture of different physical properties which may be more suitable for transport or storage than the fat fed, or (b) a chemical change consisting in a rearrangement of the fatty acids *in the molecules* of some or all of the glycerides, which in addition to changing their physical properties, would make them presumably more adaptable for use as tissue fats—for “endogenous” metabolism.

Observed changes in fats during absorption.

That fats do not pass into the chyle in exactly the form in which they occur in the food has been noted by several observers. Arnshink⁶ on feeding mutton fat to dogs found that the feces fat had a higher melting point than the food fat and drew the

⁵ An excellent example of the influence of molecular arrangement on protein utilization is reported in the recent work of Dakin and Dudley (this *Journal*, xv, p. 271, 1913) on racemized casein.

⁶ Arnshink: *Zeitschr. f. Biol.*, xxvi, p. 434, 1890.

conclusion that there was a discrimination in favor of the softer fats during absorption. Munk⁷ experimenting with a case of human chyle fistula after feeding mutton tallow found that the chyle fat had a lower melting point than the food fat, thus supplementing the findings of Arnschink. He explained the change also as being due to a discrimination in favor of the softer fats. Some results which he obtained later with the same patient indicate, however, that there is another factor. After excluding other fat from the diet, and feeding his patient cetyl palmitate, m.p. 55°, he found that the chyle contained in addition to palmitic acid (as triglyceride) about 14 per cent of combined oleic acid, also that the chyle fat had a melting point of 36°C. while tripalmitin melts at 65°C. He proved that the oleic acid could not have come from the food and must therefore have been supplied by the intestine.

Frank⁸ in feeding experiments with dogs using fats of different melting points found that the melting point of the chyle fat was generally closer to that of the body temperature of the animal than that of the fat fed. In the same paper (Experiment 11) after feeding ethyl palmitate, he found that the melting point of the chyle fatty acids was 50.5°C. (palmitic acid 63°), while their iodine number was 32.6—corresponding to an oleic acid content of 36 per cent. In Experiment 12, after feeding ethyl palmitate (other fats were excluded from the diet in both experiments) and fractionating the chyle fat, one fraction was obtained which melted at 39° and had an oleic acid content of 25 per cent⁹ (calculated from the iodine number). As in Munk's experiment the oleic acid was demonstrated not to have come from the food and was believed to have been supplied by the intestine or liver (via the bile).

From calculations based on figures for the chyle fat (per hour) of dogs given by v. Walther¹⁰ and by Munk,¹¹ Frank concluded

⁷ Munk: *loc. cit.*

⁸ Frank: *Zeitschr. f. Biol.*, xxxvi, p. 568, 1898.

⁹ It is worthy of note that an oleo dipalmitin (oleic acid content = 33.8 per cent) prepared by Kreis and Hafner (*Zeitschr. f. Untersuch. d. Nahr. u. Genussm.*, 1904, p. 605) had a melting point of 37–39°. Its calculated iodine number would be 30.4.

¹⁰ v. Walther: *DuBois Raymond's Archiv*, 1890, p. 329.

¹¹ Munk: *loc. cit.*

that the extra fat found mixed with the food fat in the chyle, was only that normally present in the chyle and was not a purposive addition. A fact which he overlooked, however, was that the dogs used by Munk and v. Walther were as a rule very much larger than his dogs and would therefore yield more chyle per hour. Recalculating his results on the more logical percentage basis, it will be seen that even if all the fat of the fasting chyle were considered as oleic acid, the fat of fasting dog chyle does not account for the amounts of oleic acid found in his experiments. For example in Experiment 11, p. 576, 27 cc. of chyle yielded 0.372 gram of fat containing 40 per cent oleic acid = 0.14 gram of oleic acid.

27 cc. of fasting dog chyle would have yielded 0.07 gram of fat (0.25 per cent of the chyle—average of Munk's and V. Walther's figures).

In Experiment 12, p. 576, 40 cc. of chyle contained 1.43 grams fat with 13 per cent oleic acid = 0.186 gram; 40 cc. of fasting dog chyle contains 0.1 gram of fat.

Since, as will be shown later, the fat of fasting dog chyle does not consist entirely of oleic acid, the fact of these additions to the chyle fat becomes the more remarkable.

Bloor,¹² after feeding pure isomannid dilaurate, found that the purified chyle fat had a melting point of 32° (pure trilaurin melts at 45–46°). The fatty acids prepared from the chyle fat had a melting point of 30°, a mean molecular weight of 211, and an iodine number of 16.5. The corresponding figures for lauric acid are: m.p., 43.6°; mean molecular weight, 200; iodine number, 0. Calculating the unsaturated fatty acids as oleic acid from the iodine number, 18 per cent of the chyle fatty acids was oleic acid. The mean molecular weight of the chyle fatty acids, assuming them to consist only of lauric and oleic acids would then have been 215.3. Since the actual mean molecular weight was 211, oleic acid was probably not the only acid added, although it was the main one. Taking into account in this experiment the amount of chyle collected and accepting the figures of Munk and v. Walther for the fat content of fasting dog chyle it cannot be denied that the added oleic acid may have had its origin in this experiment in fat normally present in the fasting chyle.

¹² This *Journal*, xi, p. 429, 1912.

Raper,¹³ after feeding cocoanut oil, which contains a considerable amount of lower fatty acids, observed the following differences between the food fatty acids and those of the chyle fat. Cocoanut oil fatty acids: mean molecular weight, 212; iodine number, 7.7. Chyle fatty acids: mean molecular weight, 236; iodine number, 19.1. The change in molecular weight was considerably greater than could be accounted for by the oleic acid added, from which (and other evidence) Raper believes that the lower fatty acids were absorbed through some other channel than the chyle.

Bloor,¹⁴ in feeding experiments with cocoanut oil, found somewhat similar differences between the cocoanut oil fed and the fat of the chyle. The cocoanut oil fed had an iodine number of 7.3 and a melting point of 26°. The chyle fat had the same melting point, but the iodine number had increased to 24°.

The few results available on this point then indicate (1) that changes in the fats may be produced during absorption; (2) that changes are probably greater than could be produced by the fat present in the normal fasting chyle.

In the work recorded below evidence is submitted of further various changes in fats during absorption which make it seem probable that the intestine is able to radically modify the composition of fats during absorption. The tendency of the changes observed is toward the production of a fat more nearly like the body fat of the animal than the fat fed. The changes appear to be purposive, since not only are they as a rule much greater than would be produced by the fats present in fasting chyle, but they vary in kind and degree with the nature of the fat fed.

EXPERIMENTAL.

The changes above recorded consist essentially in lowering of the melting point and raising of the iodine number by the addition of oleic acid. With the possible exception of the earlier work of Frank, no results are available which indicate a change in the chyle fat in the reverse direction, *i.e.*, an elevation of the melting point and lowering of the iodine number—the production of a chyle fat less liquid than the food fat.

¹³ Raper: *this Journal*, xiv, p. 117, 1913.

¹⁴ Bloor: *loc. cit.*

In the course of the study of another phase of fat absorption¹⁵ it was observed that when olive oil (mixed with various hydrocarbons) was fed to dogs, the melting point of the chyle fatty acids was generally considerably higher than that of the olive oil fed, while the iodine number was lower. A further examination of the fat of these samples of chyle was made and the data which pertain to the present discussion are given below (Table I).

TABLE I.

EXP. NO.	MATERIAL FED	VOLUME OF CHYLE	WEIGHT OF CHYLE FAT	FATTY ACIDS FROM CHYLE FAT	
				M. P.	Iodine Number
		cc.	gms.		
I	Olive oil and hydro- carbon oil.....	165	1.2	30-32	86.8
II	Olive oil and hydro- carbon oil.....	65	0.8	30	79.4
III	Olive oil and hydro- carbon jelly.....	80	1.6	27	71.7
IV	Olive oil and hydro- carbon jelly.....	75	0.5	below 20°C.	80.9
V	Olive oil and hydro- carbon oil emulsi- fied.....	179	5.9	29.5	80.3
VI	Olive oil and hydro- carbon oil emulsi- fied.....	100	4.1	30	84.0

The fatty acids of the olive oil fed had a m.p. of about 16°, and an iodine number of 86.1°.

The chyle fatty acids obtained above had with one exception in each case a much higher melting point and a lower iodine number than the fatty acids of the olive oil fed. The unused fatty acids from these experiments were united, dissolved in ether, treated with bone black until the solution was nearly colorless, then the ether evaporated and the residue dried. The melting point of the combined fatty acids was 29.5°. The acids, on fractionation from chilled petroleum ether, yielded two fractions, the first of which was crystalline and amounted to 22.5 per cent of the whole. After repeated recrystallization it gave a melting point of about 56° (a fraction of constant m.p. could not be ob-

¹⁵ Bloor: *loc. cit.*

tained), and an iodine number of 4.1. This fraction was probably a mixture of the higher saturated fatty acids.

The remaining portion, soluble in cold petroleum ether, after repeated chilling in small amounts of petroleum ether, until no more would separate, had an iodine value of 85.9 and a melting point of about 14°C. It was therefore nearly pure oleic acid.

There was obtained then after feeding olive oil whose fatty acids consisted of 96 per cent oleic acid, a chyle fat containing approximately 22.5 per cent of solid fatty acids and 77.5 per cent oleic acid and with an average melting point of 29.5°C.

As already noted, these results were obtained after feeding the olive oil in admixture with hydrocarbons and although none of the hydrocarbons were absorbed, they may have had an influence on the composition of the chyle fat. Further experiments on this point and to determine the nature of the glycerides obtained, are in progress.

In order to obtain more data regarding the changes in the fats during absorption and to determine whether there was any relationship between the amount of change and the nature (especially m.p.) of the fat fed, as well as to settle definitely whether the foreign fat found in the chyle was normally present in fasting chyle or was purposely added, experiments were conducted in which esters of pure fatty acids of various melting points were fed and the chyle fat examined.

Feeding of pure fatty acid esters and collection of the chyle.

Ethyl esters of stearic, palmitic and lauric acids were prepared by the action of their chlorides upon ethyl alcohol. The chlorides were prepared from pure fatty acids (mainly Kahlbaum's K preparations) by the method of Krafft and Bürger,¹⁶ and in the preparation of the esters were added slowly with stirring to excess of absolute alcohol. The solution was allowed to stand over night, then poured into excess of water. After thorough washing the material was ready for use. The animals (dogs) were starved for forty-eight hours before the feeding. The esters, which were all liquid at body temperature, were given by stomach tube and the feeding was followed by about 50 grams of bread which had been rendered fat-free by boiling with alcohol.

¹⁶ Krafft and Bürger: *Ber. d. deutsch. chem. Gesellsch.*, xvii, p. 1378, 1884.

The operations for the insertion of the cannula into the thoracic duct (for the earlier of which I am indebted to Doctor W. M. Marriott of this laboratory), were performed under ether anaesthesia. Special efforts were made to make the shock of operation as light as possible and to bring the animal after the operation into a condition as nearly normal as possible for the experiment. The operations were done with aseptic precautions making a small wound; and by the use of a paraffined cannula of narrow lumen (2 mm.), clotting was prevented without the use of special devices. As soon as the cannula was safely in the duct and the wound closed, the animal was removed to a padded table, covered warmly and allowed to recover from the anaesthetic. The return to consciousness was always followed by an improvement in the fat content of the chyle. Collection of chyle was continued as long as convenient. The animals rested quietly most of the time. If they became restless they were removed from the table and allowed to walk around for a while, after which they were returned to the table and generally went to sleep. Water was given as often as desired. As previously mentioned, the chyle was occasionally found to be flowing on the second day and another experiment could be made with the same animal. The cannula generally dropped out on the third day and the animal in almost all cases made a good recovery.

The chyle was collected in a vessel containing a little dry magnesium sulphate to prevent clotting and when collection was complete it was transferred to a separatory funnel, shaken well with ether and the mixture allowed to stand over night. The extracted chyle, now clear, was run off, evaporated to dryness on a water bath, powdered and again extracted by boiling out two or three times with ether. These ether extracts were added to the first and the whole evaporated to dryness. The essential points of the experiments are as follows:

ETHYL STEARATE. Dog, weight 10 kgm., a fat female, had been used in a similar experiment the day before. The chyle was still flowing and was clear. At 10 a.m. she was fed 7.3 grams of the pure ester of which a considerable portion was vomited shortly afterwards. The volume of chyle was not noted. Total chyle fat collected, 0.3 gram. The fat was saponified and the fatty acids separated from unsaponifiable matter and purified with bone black.

Melting point of the fatty acids, 45°C.

Iodine number, 56.21—containing therefore 62.5 per cent oleic acid.

Mean molecular weight, 285.

ETHYL PALMITATE. Experiment I. The dog, a female, weight 25 pounds, thin and active, was fed 20 grams of ethyl palmitate at 8.30 a.m. The operation was complete and collection was begun at 11.45. Collection was continued for ten hours with a total yield of chyle of 147 cc., containing 1.3 grams of fat—0.9 per cent.

Melting point of the fat, 55°.

Iodine number, 66.9.

Experiment II. Next morning the chyle was still flowing and clear, so another feeding of 20–25 grams of ester was given together with 50 grams of coagulated egg white, at 10.30 a.m. By 12 o'clock the chyle had begun to appear milky. Collection was then begun and continued until 7.45 p.m. Total chyle collected, 79 cc., containing 1.6 grams of fat—2 per cent.

Melting point of the fat, 57°C.

Iodine number, 52.3.

The chyle fat from the two palmitate experiments was united.

Average melting point, 56°. Average iodine number, 59.6.

It was fractionated from ether in the cold, yielding two main fractions of which the first and largest—1 gram—after several recrystallizations, yielded well formed crystals with a melting point of 61°C. and an iodine number of 7.6. It was probably nearly pure tripalmitin. The second fraction, from which no other substance could be separated was liquid at room temperature. Its iodine number was 62.5, corresponding to an oleic acid content of 69.4 per cent. (This value is suggestively close to that of a dioleopalmitin oleic acid, 65.7 per cent.)

ETHYL LAURATE: A female dog weighing 20 pounds, in fair condition, was fed 18 grams of ethyl laurate, together with 50 grams of fat-free bread and 10 grams of glycerin, at 8.30 a.m. Collection of chyle was begun at 1.15. From 1.15–3.15, 58 cc. containing but little fat were collected and treated separately (see below). At 3.15 the chyle was becoming richer in fat and continued of good color until 9.45 p.m., when collection was stopped. This portion (II), total 106 cc., was extracted separately (see below). Next morning the chyle was still flowing and of a good white color. Collection was begun again at 8.30. At about 11.00 a.m. it began to lose its white color and 9 grams more of ethyl laurate were fed through a stomach tube. Collection was continued until 9.45 p.m. The chyle from this period, total 131 cc., was treated separately (portion III). The extractions of the separate portions were made as in the other experiments.

Portion I. 58 cc.; total fat, 0.21 gram, 0.36 per cent; m.p. 22–24°C.

Portion II. 106 cc.; total fat, 1.82 grams, 1.71 per cent; m.p. 23–24°C.

Portion III. 131 cc.; total fat, 1.15 grams, 0.88 per cent; m.p. 30°C.

Iodine number, 56.35.

Portion IV. The chyle from the above three portions was evaporated to dryness, powdered and extracted with hot ether. Weight of extract 0.57 gram.

Total laurate chyle collected, 295 cc., containing 3.75 grams of fat—1.27

per cent. Iodine number of the whole chyle fat, 44. Attempts were made to fractionate this fat but without success.

The essential facts of the ester experiments are collected in table II. The results of Munk and Frank already cited (pp. 520-521) are added for comparison.

From the results given it may be seen:

1. That the amount of "oleic acid" in the chyle fat is generally much greater than could be accounted for by the fat of fasting chyle, accepting the average value 0.25 per cent already quoted (p. 521) and supposing it to be entirely oleic acid.

2. That there is a parallelism between the melting point of the fatty acids fed and the amount of "oleic acid" added—the higher the m.p. of the fatty acid, the more oleic acid.

3. That the fat of the fasting chyle is not always entirely, or even mainly, oleic acid, as may be seen from the laurate experiments. Since the chyle in portion I has the lowest fat content it should contain most of the fasting chyle fat, and if this fat were oleic acid, the iodine number should be highest of the three portions, while it is actually lowest.

4. That there is a marked similarity in the results of Frank's experiments with ethyl palmitate and those reported in this paper. The chyle fat of both yielded two main fractions, one of which was undoubtedly tripalmitin and the other with an iodine number and melting point close to those of mixed glycerides of palmitin and olein.

It seemed of interest to know also whether any change would be produced in a fat with an already high iodine number and low melting point. For this purpose cod liver oil, iodine number 148, was fed to a dog, the chyle collected and the chyle fat examined as in the earlier experiments.

Total chyle, 76 cc.

Fat of chyle, 1.05 gram—1.4 per cent.

Iodine number, 118.

The iodine number was reduced from 148 to 118 during the absorption.

Here for completeness may be mentioned again the results of experiments with cocoanut oil which have already been mentioned above (p. 522), as examples of a change of a somewhat different nature.

TABLE II.

MATERIAL FED	VOLUME OF CHYLE	CHYLE FAT			WEIGHT OF OLEIC ACID IN CHYLE FAT (CALC.) <i>grams.</i>	WEIGHT OF ACID IN SAME VOL. OF FASTING CHYLE <i>grams.</i>	REMARKS
		Weight <i>grams.</i>	M. P. <i>deg. C.</i>	Iodine Num- ber			
Ethyl stearate.....	cc.	0.3	45	56.2	0.2	Volume of chyle not noted.	
Ethyl palmitate							
Exp. I.....	147	1.3	55	66.9	1.0		0.36
Exp. II.....	79	1.6	57	52.3	0.97		0.19
On Fractionation						Probably tripalmitin, m.p., 65.5. Possibly di-oleopalmitin, iodine no. 59.2 (calc.).	
Fraction I.....			61	7.6			
Fraction II.....			below 20	62.5			
Ethyl laurate.							
Portion I.....	58	0.21	22-24	32.8	0.08	0.14	
Portion II.....	106	1.82	22-24	44.08	0.9	0.26	
Portion III.....	131	1.15	30	56.4	0.75	0.33	
Cetyl palmitate (Munk).....	1200	3.97	36		0.57*	1.2†	
Ethyl palmitate (Frank)							
Exp. 11.....	27	0.37	51	32.6	0.14	0.07	
Exp. 12.....	40	1.43			0.19	0.10	
On fractionation							
Fraction I.....			39	22.7		Values for an oleo-dipalmitin are m.p., 37-38; Iodine no. 31.4 (calc.). Probably tripalmitin.	
Fractions II and IV (av.)			62	4.0			

* The oleic acid was determined by separation as lead salt and the value is probably low.

† Calculated from his own determination of fat in human hunger chyle (0.1 per cent).

EXPERIMENT I (Raper): The cocoanut oil fed had an iodine number of 7.7, and a mean molecular weight of 212. The chyle fat collected had an iodine value of 19.1, and a mean molecular weight of 236.

EXPERIMENT II (Bloor): Cocoanut oil (fed together with hydrocarbon oil). Iodine number, 7.3; m.p., 26°; chyle fat iodine number, 24; m.p., 26°.

The changes in cocoanut oil consist in the addition of "oleic acid" without a change in the melting point.

SUMMARY AND CONCLUSIONS.

Evidence is presented of changes in fat during absorption as follows:

1. A lowering of the melting point of high melting point fats by the addition of an unsaturated fatty acid, probably oleic acid. The addition is proportional to the melting point of the fatty acid fed.

2. An elevation of the melting point and lowering of iodine number of a low melting point fat (olive oil) by the addition of saturated fatty acids.

3. Addition of "oleic acid" together with a change in the mean molecular weight of the fatty acids, without change of melting point in a fat which consists mainly of glycerides of saturated fatty acids (cocoanut oil).

4. Lowering of the iodine number of a fat (cod liver oil) which contains a large percentage of glycerides of highly unsaturated fatty acids.

The intestine appears to have the power to modify radically the composition of the fats during absorption. The changes are apparently purposive in that they vary in kind and degree with the nature of the fat fed and also show in general a *tendency* toward the production of a uniform chyle fat, presumably the characteristic body fat of the animal.

In the preceding paper of this series one reason was suggested for the peculiar mechanism of fat absorption—that it serves to exclude undesirable fat-like substances such as the petroleum hydrocarbons, etc. The observations presented above, suggest a second—that the mechanism serves to permit adaptive changes in the fats during absorption.

Work is being continued along similar lines.

THE HEXONE BASES OF CASEIN.

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In our preliminary description of the method for analysis of proteins by determination of the chemical groups characteristic of the different amino-acids, we published an analysis of casein.¹ The results agreed quite well with those previously obtained by other authors with the Kossel method for determining the bases of proteins. Consequently, although our method was improved before its final publication, we did not repeat the casein analysis. The discrepancy, noted in the preceding article, between the free amino nitrogen of casein, and the lysine content previously determined, rendered a repetition of the nitrogen distribution in this protein desirable. We have, therefore, determined the bases by the method of Kossel and Patton, as modified by Osborne, Leavenworth, and Brautlecht,² and have also redetermined the bases and nitrogen distribution by our previously published method of group analysis.

The most significant differences between our present results and previous ones occur in the lysine. The percentages of the casein nitrogen previously found in the lysine were 6.66 to 7.24³ by Kossel's method and 7.86 by our own. By exercising particular care in the Kossel method we have now obtained 9.36 per cent of the casein nitrogen in the form of lysine weighed as the analytically pure picrate. Our group determination method gave 10.3 per cent, and we believe that this figure is even more nearly correct, as the amount of lysine picrate which one can crystallize represents necessarily the minimum amount present. For arginine the results are practically the same, 7.4 to 7.8 per cent of the total nitrogen,

¹ *Ber. d. deutsch. chem. Gesellsch.*, xliii, p. 3179, 1910.

² *Amer. Journ. of Physiol.*, xxiii, p. 183, 1908.

³ *Ibid.*

as those previously obtained by both methods. The histidine results are a little higher than previously, but not to a marked extent.

The source of error in our own former results for lysine lay in the cystine determination. The lysine is estimated from the total amino nitrogen of the bases precipitated by phosphotungstic acid, after the cystine nitrogen has been subtracted. The cystine was estimated from the amount of organic sulphur precipitated with the bases. The original form of the method, however, made the cystine figures liable to error from the fact that sulphates could be dissolved from a glass flask used in one stage of the operation. Although this source was recognized⁴ and a correction, determined from controls, attempted for it, the results for cystine were nevertheless much too high, those for lysine being consequently low. In the form to which the method was modified before being published in detail in this *Journal*,⁵ the above source of error in the cystine and lysine determinations was eliminated.

In the determination by the picrate method, as usually performed, it appears that the most probable source of loss lies in the decomposition of lysine phosphotungstate with barium hydrate. In this operation one insoluble precipitate (lysine phosphotungstate) is transformed into another (barium phosphotungstate), a process the completeness of which is necessarily difficult to judge. Moreover, the bulky barium phosphotungstate has marked adsorptive properties, so that even skill and experience might not insure against loss from this source. In working out the details of the group determination method, we noticed that several per cent of the total nitrogen of the protein could be lost from the base fraction through adsorption or occlusion by the barium precipitate. We therefore made a practice of reducing this loss to a minimum by completely dissolving the phosphotungstates of the bases with alkali, and precipitating the barium phosphotungstate in a dilute solution.⁶ In the more successful of our present determinations by Kossel's method we have dissolved the lysine phosphotungstate in ammonia and diluted the solution to a large volume before treating with barium hydrate.

⁴ *Ber. d. deutsch. chem. Gesellsch.*, xliii, p. 3177, 1910, footnote.

⁵ This *Journal*, x, p. 16, 1911.

⁶ *Ibid*, x, p. 25, 1911.

First analysis by Kossel's method.

In this determination the casein was completely hydrolyzed with hydrochloric acid and all the bases were precipitated with phosphotungstic acid. The precipitate was decomposed with barium hydrate, using a large volume of solution and a mechanical stirrer to make the decomposition quantitative and keep loss by adsorption as low as possible. The bases were then separated by Osborne, Leavenworth, and Brautlecht's modification of Kossel and Patton's method. The details follow.

Forty grams of Merck's "Casein nach Hammarsten" were boiled thirty hours with 400 cc. of 20 per cent hydrochloric acid. The solution was then diluted to 1 liter, and three samples of 5 cc. each removed for Kjeldahl determinations. The amounts of $\frac{N}{10}$ acid neutralized were 18.70, 18.67, and 18.65 cc., the average indicating 5.15 grams of nitrogen in the remaining 985 cc. of solution.

The latter was diluted with water to 2 liters, and the bases were precipitated with 150 grams of purified phosphotungstic acid. After two days the precipitate was filtered with suction and washed with a solution of 2.5 per cent phosphotungstic acid in 5 per cent sulphuric acid until the chloride reaction disappeared from the filtrate. The precipitate was then suspended in 5 liters of water and thoroughly stirred with a machine while an excess of barium hydrate solution was added. The stirring was then continued for about two hours. The filtrate from the barium phosphotungstate was concentrated in a vacuum, the ammonia being driven off in the process. The excess barium was then removed with carbon dioxide, and the solution concentrated to 1000 cc. Twenty-five cc. were removed for analyses, which gave a total nitrogen of 1.176 grams, or 22.8 per cent of the entire casein nitrogen, and an amino nitrogen of 0.754 gram, or 14.63 per cent.

The remaining 975 cc. of the solution, containing the basic portion of 5.02 grams casein nitrogen, were concentrated in a vacuum, and the histidine precipitated as described by Osborne, Leavenworth, and Brautlecht.

The histidine solution was brought to 100 cc. volume.

2.000 cc. for NH_2 determination gave 2.51 cc. N gas at 21°, 774 mm.

10.00 cc. for Kjeldahl determination neutralized 14.92 cc. of $\frac{N}{10}$ acid.

Amino nitrogen in histidine solution, 0.0723 gram.

Total nitrogen in histidine solution, 0.2090 gram = 4.16 per cent of total casein nitrogen.

Ratio (total nitrogen) : (amino nitrogen) = 2.89

Ratio calculated for histidine . = 3.00

The arginine solution was also brought to 100 cc.

2.000 cc. for NH_2 determination gave 3.41 cc. N gas at 23°, 764 mm.

5.00 cc. for Kjeldahl determination neutralized 13.44 and 13.50 cc. of $\frac{N}{10}$ acid.

Amino nitrogen in arginine solution, 0.0962 gram.

Total nitrogen in arginine solution, 0.3770 gram = 7.51 per cent of total casein nitrogen.

Ratio (total nitrogen) : (amino nitrogen) = 3.92

Ratio calculated for arginine = 4.00

The filtrate from the arginine was freed from barium and silver, and the lysine reprecipitated with phosphotungstic acid at 1 liter volume. The lysine phosphotungstate was dissolved in 2 liters of dilute ammonia and freed from phosphotungstic acid with barium hydrate. The ammonia was boiled off in a vacuum, the excess barium removed with carbon dioxide, and the lysine solution brought to 100 cc.

1.000 cc. for NH_2 determination gave in 0.5 hour 8.28 cc. N gas at 20° , 746 mm.

5.00 cc. for Kjeldahl determination required 16.48 cc. of $\frac{\text{N}}{10}$ acid.

3.00 cc. for Kjeldahl determination required 9.71 cc. of $\frac{\text{N}}{10}$ acid.

Amino nitrogen in lysine solution, 0.462 gram = 9.21 per cent of total casein nitrogen.

Total nitrogen in lysine solution, 0.461 to 0.460 gram.

Ninety cc. of the solution were concentrated to 40 cc., heated to boiling and 1 equivalent (3.39 grams) of picric acid was dissolved in the hot solution. The lysine picrate obtained weighed, when dried to constant weight, 5.063 grams, equivalent to 0.378 gram of lysine nitrogen in the 90 cc. of the original solution, or 0.422 gram in the total solution, the latter amount being 8.39 per cent of the nitrogen of the casein. Allowing for the solubility of lysine picrate in water at 20° (0.5 gram per 100 cc.) increases the lysine picrate to 5.26 grams, and the percentage of lysine nitrogen in casein to 8.70.

The lysine which crystallized was analytically pure.

ANALYSIS: 0.1532 gram substance; 20.6 cc. N at 22° , 748 mm. (nitrous acid method).

	Calculated for $\text{C}_6\text{H}_{10}\text{O}_7(\text{NH}_2)_2 \cdot \text{C}_6\text{H}_3\text{N}_3\text{O}_7$:	Found:
Amino nitrogen.....	7.47	7.45

Of the total amount of nitrogen in the lysine fraction, 92 per cent was recovered as pure lysine picrate, or 95 per cent if the solubility of the picrate is taken into account. This indicates strongly that in casein which has been *completely* hydrolyzed and freed from ammonia there are, aside from arginine, histidine, lysine, and the cystine not destroyed by the hydrolysis, no amino-acids precipitated by phosphotungstic acid under the usual conditions, *i.e.*, at room temperature, in the presence of mineral acid of $\frac{\text{N}}{1}$ concentration and a moderate excess of phosphotungstic, the concentration of the proteolytic products being about 2 per cent.

It will be noted that all the non-amino nitrogen of the total

phosphotungstate precipitate is accounted for by two-thirds the histidine nitrogen + three-fourths the arginine. Of the 14.6 per cent amino nitrogen in the first precipitate, however, the amount recovered in the histidine, arginine, and lysine fractions respectively was $1.19 + 1.88 + 9.21 = 12.28$ per cent. The 2.3 per cent loss is doubtless in part due to adsorbed lysine, but a portion is probably due to mono-amino-acids which were not completely removed from the first phosphotungstate precipitate. The latter was washed on a suction funnel in the ordinary manner, and not triturated in the way which we have found necessary for a quantitative washing of these precipitates. As the bases were reprecipitated separately before they were determined, however, the final results are not affected.

Second analysis by Kossel's method.

In this analysis we attempted to keep possible losses from adsorption by barium phosphotungstate at a minimum. The preliminary precipitation of all the bases with phosphotungstic acid was left out, and the regular technique of Osborne followed for the precipitation of arginine and histidine directly from the solution of all the amino-acids by means of silver nitrate and barium hydrate. The lysine was then precipitated as phosphotungstate. In the decomposition of the latter, it was completely dissolved with ammonia and diluted before treatment with barium hydrate.

The casein was hydrolyzed as in the first analysis and the solution diluted to 1 liter. Three samples, of 5 cc. each, taken for Kjeldahl determinations required 19.55, 19.65, and 19.75 cc. of $\frac{N}{10}$ acid, the average indicating 5.42 grams of nitrogen in the remaining 985 cc. of solution. The latter was freed as completely as possible from hydrochloric acid by concentration under diminished pressure. The residue was taken up in water, made alkaline with barium hydrate, and the ammonia was driven off by concentrating again under diminished pressure. The solution was then acidified with nitric acid, and the remaining hydrochloric acid removed with silver nitrate. The filtrate from the silver chloride was brought to a volume of 1 liter, and the histidine and arginine precipitated with excess silver nitrate and barium hydrate. The solution of the two bases was brought to 250 cc., from which 12 cc. were taken for amino and Kjeldahl determinations. These showed 0.707 gram nitrogen and 0.211 gram amino nitrogen present. The remaining 238 cc. of the solution, containing the arginine-histidine fraction of 5.16

grams of casein nitrogen, were submitted to the Osborne modification of Kossel and Patton's technique for separation of the two bases by precipitation of the histidine with mercuric sulphate.

The histidine solution was brought to 100 cc.

2.000 cc. for NH_2 determination gave (1) 2.59 cc. N gas at 17° , 779 mm.;

(2) 2.69 cc. at 25° , 765 mm.

3.00-cc. portions for Kjeldahl determination required 5.00 cc. each of $\frac{\text{N}}{16}$ acid.

Amino nitrogen in histidine solution, 0.0758 to 0.0752 gram; average, 0.0755 gram.

Total nitrogen in histidine solution, 0.2330 gram = 4.51 per cent of the casein nitrogen.

Ratio (total nitrogen) : (amino nitrogen) = 3.09

Ratio calculated for histidine = 3.00

The arginine solution was also brought to 100 cc.

2.000 cc. for NH_2 determination gave 3.60 cc. N gas at 17° , 770 mm.

3.00 cc. for Kjeldahl required 8.60 to 8.65 cc., average 8.63 cc., $\frac{\text{N}}{16}$ acid.

Amino nitrogen in arginine solution, 0.1052 gram.

Total nitrogen in arginine solution, 0.4040 gram = 7.83 per cent of the casein nitrogen.

Ratio (total nitrogen) : (amino nitrogen) = 3.84

Ratio calculated for arginine = 4.00

From the filtrate of the first arginine-histidine precipitate the lysine was precipitated as phosphotungstate in the usual manner. The precipitate was redissolved in 2 liters of dilute ammonia and the phosphotungstic acid removed by addition of barium hydrate. The filtrate from the barium phosphotungstate was freed from ammonia by concentration under diminished pressure, from barium by means of carbon dioxide, and was then brought to 100 cc. Analysis showed that 0.605 gram of amino nitrogen was present. To 75 cc. of the solution one equivalent (3.75 grams) of picric acid was added, and the mixture was heated until solution was complete. After two days' standing 4.69 grams of lysine picrate, equivalent to 6.26 grams for the entire 100 cc. of solution, crystallized analytically pure. The amount of lysine nitrogen calculated from the picrate is 0.467 gram, or 8.62 per cent of the total nitrogen of the casein.

ANALYSIS: 0.1433 gram substance; 18.85 cc. N gas at 19.5° , 760 mm. freed by nitrous acid method.

	Calculated for $\text{C}_6\text{H}_{10}\text{O}_2(\text{NH}_2)_2 \cdot \text{C}_6\text{H}_2\text{N}_3\text{O}_7$:	Found:
Amino nitrogen.....	7.47	7.50

The filtrate from the above main crop of picrate was acidified with sulphuric acid, freed from picric acid with ether, and treated again with phosphotungstic acid in a volume of 100 cc. A second crop of lysine phosphotungstate was obtained, which eventually yielded 0.40 gram of pure lysine picrate.

ANALYSIS: 0.0327 gram substance; 4.35 cc. N gas at 24° , 760 mm., by the nitrous acid method with micro-apparatus.

	Calculated for $C_6H_{10}O_2(NH_2)_2 \cdot C_8H_8N_2O_7$	Found:
Amino nitrogen.....	7.47	7.42

This second crop of lysine brings the total lysine nitrogen up to 0.507 gram, equal to 9.36 per cent of the casein nitrogen.

Analysis by the nitrogen distribution method.

Ten grams of casein were boiled twenty-four hours with 200 cc. of 20 per cent hydrochloric acid.⁷ The acid was driven off as completely as possible by concentrating under diminished pressure, and the solution was brought to 150 cc. Three samples of 5 cc. each required 35.05, 35.20, and 35.05 cc., an average of 35.10 cc., of $\frac{N}{10}$ acid in Kjeldahl determinations. For the nitrogen distribution* 50 cc. of the solution, containing 0.491 gram of nitrogen, were taken. The analysis was performed as described in the paper on the method.⁸

Ammonia. The amount of $\frac{N}{10}$ acid neutralized was 36.00 cc., equivalent to 0.0504 gram of ammonia nitrogen.

Melanine. The amount of $\frac{N}{10}$ acid neutralized was 4.50 cc., equivalent to 0.0063 gram of melanine nitrogen.

Cystine. The weight of barium sulphate was 0.0035 gram, equivalent to 0.0010 gram of cystine nitrogen in all.

Arginine. The volume of $\frac{N}{10}$ acid neutralized was 6.50 cc., equivalent to 0.0364 gram of arginine nitrogen.

Total nitrogen of the bases. The amount of $\frac{N}{10}$ acid neutralized in the Kjeldahl determination was 35.65 cc. Added to the amount neutralized in the arginine determination, this gives 42.15 cc., equivalent to 0.1181 gram of nitrogen, or 24.27 per cent of the total nitrogen of the casein.

Amino nitrogen of the bases. Two-cc. portions of the solution of the bases gave, in the micro-apparatus, 5.02 cc. of nitrogen gas at 23°, 778 mm., and 4.94 cc. at 20°, 778 mm., indicating respectively 0.0722 and 0.0719 gram of amino nitrogen in the bases, equivalent to 14.6 per cent of the total nitrogen of the casein.

Amino nitrogen of the filtrate. Duplicates gave each 31.40 cc. nitrogen gas at 20°, 781 mm., equivalent to 0.274 gram of amino nitrogen.

Total nitrogen of the filtrate. The amounts of $\frac{N}{10}$ acid neutralized were 36.65 and 36.90 cc., the average, 36.78 cc., indicating 0.309 gram of nitrogen in the filtrate.

The results are tabulated on the following page.

⁷ Van Slyke: Conditions for Complete Hydrolysis of Proteins, this *Journal*, xii, p. 295, 1912.

⁸ This *Journal*, x, p. 15, 1911.

	NITROGEN	TOTAL NITROGEN
	grams	per cent
Ammonia.....	0.0504	10.27
Melanine.....	0.0063	1.28
Cystine.....	0.0010	0.20
Arginine.....	0.0364	7.41
Histidine.....	0.0305	6.21
Lysine.....	0.0506	10.30
Amino nitrogen of filtrate.....	0.2740	55.81
Non-amino nitrogen of filtrate.....	0.0350	7.13
Total nitrogen recovered.....	0.4842	98.61

SUMMARY.

The following figures for the bases of casein were obtained by the method of Kossel, and by the author's nitrogen distribution method. The figures represent percentages of the total nitrogen of the casein.

	KOSSEL'S METHOD		NITROGEN DISTRIBUTION METHOD (Uncorrected for solubility of bases)
	First Analysis	Second Analysis	
Histidine.....	4.16	4.51	6.21
Arginine.....	7.51	7.83	7.41
Lysine.....	8.70	9.36	10.30

It appears probable that low results for lysine (7 per cent) obtained previously by the Kossel method were due to adsorption or occlusion of lysine by barium phosphotungstate, a source of error which we attempted to avoid, especially in the second Kossel analysis. That the lysine crystallized as picrate represents the entire amount present is improbable, and the lysine content obtained by the nitrogen distribution method is doubtless more nearly correct.

From the data in this and previous papers,⁹ which permit a comparison of results by the two methods, it appears that the nitrogen distribution method is somewhat more reliable than the Kossel method for lysine determination in proteins, that both methods are quite accurate for arginine, and that the Kossel-Patton method, as modified by Osborne, Leavenworth, and Brautlecht, gives more consistent results for histidine.

⁹ This Journal, x, p. 16, 1911.

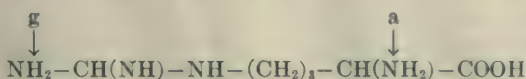
THE NATURE OF THE FREE AMINO GROUPS IN PROTEINS.

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The presence of basic groups in the proteins has long been assumed because of the ability of the proteins to neutralize acids. The specific nature of these basic groups appears to have been first indicated by work on the protamines in Kossel's laboratory. These simplest proteins are all unusually rich in one or more of the hexone bases, arginine, histidine, and lysine, and are also markedly basic, forming salts of constant composition with sulphuric acid. Goto¹ found that clupeine, which contains a large amount of arginine, binds approximately 1 equivalent of sulphuric acid for each molecule of arginine. Arginine contains two amino groups, one (*a*) in the α -position to the carboxyl group, the



other (*g*) in the guanidine nucleus, and it was uncertain which of these was free in the clupeine molecule. The point was settled by Kossel and Cameron² in favor of the guanidine group. They nitrated the free amino groups of clupeine under conditions which avoided hydrolysis of the protein. The nitroclupeine was then hydrolyzed, and nitroarginine obtained from it. The amino group in this nitroarginine could be determined by the nitrous acid method. As the guanidine group does not react with nitrous acid, the amino group freed by hydrolysis was evidently the α -group. The other

¹ *Zeitschr. f. physiol. Chem.*, xxxvii, p. 114, 1902.

² *Ibid.*, lxxvi, p. 457, 1912.

amino group, free in the intact protamine, is therefore in the guanidine nucleus.

Other evidence indicated that, in some proteins at least, one of the two amino groups of lysine, $\text{NH}_2 \cdot (\text{CH}_2)_4 \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH}$, is also free. Skraup³ found that after casein, gelatin, and serum globulin had been treated with nitrous acid, no lysine could be obtained from them. Similar results were obtained by Levites.⁴ Van Slyke demonstrated in edestin and egg albumin the presence of definite amounts of free amino nitrogen, which, though small, could be determined by the nitrous acid method, and pointed out the fact that, considering the results of Levites and Skraup, it was probable that one of the amino groups of lysine furnished a large part of the free amino nitrogen of the proteins.⁵ Since then, having determined the lysine contents of a number of protein preparations in this laboratory, we have also determined their free amino nitrogen. The results, reported in detail in the present paper, were published in a preliminary abstract in May, 1912.⁶ They showed that the free amino nitrogen of the native proteins is approximately one-half of the lysine nitrogen, indicating that one of the two amino groups of lysine is free in the protein molecule. At the time of our preliminary report the paper by Kossel and Cameron also appeared.⁷ They settled the location of the free amino group of arginine in clupeine, as mentioned above, and also showed that clupeine, which contains no lysine, gave off no nitrogen when treated with nitrous acid to determine the free amino groups. Cyprinine and sturine, which contain lysine, showed considerable free amino nitrogen. Later Kossel and Gawrilow⁸ performed the formol titration of Sørensen on hordein, zein, and several protamines, and found that the protamines containing lysine revealed amino groups by the formol method, while those which contained none, as well as zein, which also contains none, revealed no amino groups. No quantitative relations were ascer-

³ *Ann. d. Chem., cccli*, p. 379, 1906.

⁴ *Biochem. Zeitschr.*, xx, p. 224, 1909.

⁵ *This Journal*, ix, p. 196, 1911.

⁶ *Proc. Soc. Exp. Biol. and Med.*, May 15, 1912.

⁷ *Loc. cit.*

⁸ *Zeitschr. f. physiol. Chem.*, lxxxi, p. 274, 1912.

tained, however, between the lysine and free amino nitrogen contents of the proteins.⁹

In our preliminary report we gave determinations of free amino nitrogen on several native proteins, and quoted those previously published from this laboratory on proto- and heteroalbumose. The native proteins showed, as stated above, free amino nitrogen equal to approximately half their lysine nitrogen. In the hetero- and protoalbumose we found more of the total nitrogen in the form of free amino nitrogen than is calculated by halving the lysine nitrogen. This result was to be expected, as the hydrolytic cleavage from which the albumoses result sets free α -amino groups from the peptide linkings into which they are condensed in the native proteins.

We have in the meantime analyzed, in addition, gliadin, repeated with present technique the amino determination performed several years ago on the albumoses, and confirmed the results reported for the native proteins, except casein. The amount of free amino nitrogen in casein (3.4 per cent of the total nitrogen) was, through an error, calculated too low. The correct figure is 5.5 per cent. The former, incorrect figure was almost exactly one-half the lysine nitrogen previously determined in casein. The lysine content of casein had been determined some years before by the picrate method, but in the case of casein, unlike most of the other proteins, the estimation had not been checked by our group determination method in its present form. The hexone base content of casein was, therefore, carefully redetermined by both the Kossel and the group determination methods. The results are recorded in the preceding paper. The correct lysine nitrogen was found to be, as in the other proteins, nearly twice the free amino nitrogen.

For the hemocyanin preparation we thank Dr. C. L. Alsberg of the Bureau of Chemistry; for the hemoglobin, Dr. Butterfield of this Institute, and for the zein, Dr. Thomas B. Osborne of New Haven.

⁹ "Im allgemeinen scheinen die lysinreicheren Protamine auch reicher an formitirierbarem Stickstoff zu sein, doch sind die bisher vorliegenden Analysen noch nicht zahlreich genug, um hierüber zu entscheiden."

EXPERIMENTAL.

Methods. The proteins were brought into solution in 2 to 4 per cent concentration, using when necessary acetic acid or sodium carbonate in the cold to assist the process. There was no evidence of the occurrence of any hydrolysis during the preparation of the solutions. These were analyzed immediately after they were made up. When they were allowed to stand several hours, only slight increases in the amino nitrogen were noted. All determinations were made in the standard size amino apparatus described in this *Journal*, xii, p. 275, or in the micro-apparatus described in xvi, p. 121. The mixtures were shaken constantly with a motor during each determination, octyl alcohol being used to prevent foaming. The proteins, or deaminized proteins, are precipitated as soon as they are mixed with the nitrous acid solution. When the mixture is kept well stirred by shaking, however, the precipitation does not appear to influence the results, which were uniformly definite and constant. That the amino nitrogen thus determined represents amino groups free in the protein, none of the latter being hydrolyzed by the nitrous acid, is indicated by two facts:

1. Peptides of varied composition and containing up to fourteen amino-acids in the molecule have been analyzed by our method and found to give theoretical results.¹⁰

2. The evolution of nitrogen is complete inside of twenty or thirty minutes, following practically the course found in analysis of lysine,¹¹ of which the ω -NH₂ group reacts somewhat more slowly than the α -groups of the amino-acids in general. We do not believe that any part of the amino nitrogen determined comes from acid amide groups in the protein molecule. As determined by analysis of asparagine and acetamide, acid amide groups give off no nitrogen at all when treated with nitrous acid under the conditions of the determination.

Casein. Three grams of Hammarsten casein (air-dried) were dissolved in 100 cc. of water with 0.375 gram of sodium carbonate. This amount of carbonate is sufficient to dissolve the casein without rendering the solution alkaline, and autohydrolysis occurs only at a very slow rate.

¹⁰ Abderhalden and Van Slyke: *Zeitschr. f. physiol. Chem.*, lxxiv, p. 505, 1911.

¹¹ Van Slyke: this *Journal*, xii, p. 275, 1912.

Kjeldahl nitrogen: 5-cc. portions; 14.80 cc. of $\frac{N}{10}$ HCl (average of 3 determinations), indicating 8.29 mgm. of nitrogen in the 2 cc. of solution used for amino determination in the micro-apparatus. In this table the uncorrected as well as corrected results are given to show the magnitude of the correction for reagents, and its variation with the reaction time. In the subsequent tables only corrected results are given.

Amino nitrogen: 2-cc. portions.

DURATION OF REACTION WITH NITROUS ACID	NITROGEN GAS EVOLVED		TEMPERATURE	PRESSURE	AMINO N	PER CENT OF TOTAL N AS FREE NH ₂
	Volume read	Corrected for reagents				
min.	cc.	cc.	deg. C.	mm.	mgm.	
10	0.86	0.76	22	756	2.130	5.14
15	0.92	0.80	23	756	2.230	5.38
20	0.96	0.82	23	756	2.285	5.51
30	0.98	0.82	23	756	2.285	5.51

After the solution had stood forty-eight hours at room temperature the proportion of nitrogen as free NH₂ had increased to 5.84 per cent (thirty-minute reaction), indicating an appreciable, but very slow, autohydrolysis.

Gelatin. Kjeldahl nitrogen: 10-cc. portions; 24 cc. $\frac{N}{10}$ HCl. Total nitrogen in 10 cc., 33.6 mgm.

Amino nitrogen: 10-cc. portions.

DURATION OF REACTION	N GAS	TEMPERATURE	PRESSURE	AMINO N	PER CENT OF TOTAL N AS FREE NH ₂
min.	cc.	deg. C.	mm.	mgm.	
10	1.80	23	762	1.026	3.05
30	1.90	28	762	1.048	3.12
30	1.90	28	762	1.048	3.12

Or hemoglobin. Solution I. Kjeldahl nitrogen: 10-cc. portions; 12.93 cc. $\frac{N}{10}$ HCl; 18.10 mgm. N. Solution II. Kjeldahl nitrogen: 10-cc. portions; 10 cc. $\frac{N}{10}$ HCl; 14.01 mgm. N.

Amino nitrogen: 10-cc. portions.

NO.	DURATION OF REACTION	N GAS	TEMPERATURE	PRESSURE	AMINO N	PER CENT OF TOTAL N AS FREE NH ₂
	min.	cc.	deg. C.	mm.	mgm.	
Ia	10	1.80	18	760	1.033	5.70
Ib	10	1.80	18	760	1.033	5.70
IIa	30	1.50	26	758	0.825	5.89
IIb	30	1.60	26	758	0.881	6.29

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Edestin. Solution I. Kjeldahl nitrogen: 10-cc. portions; 24 cc. $\frac{N}{16}$ HCl; 37.60 mgm. N. Solution II. Kjeldahl nitrogen: 10-cc. portions; 19.33 cc. $\frac{N}{16}$ HCl; 27.06 mgm. N.

Amino nitrogen: 10-cc. portions.

NO.	DURATION OF REACTION	N GAS	TEMPERATURE	PRESSURE	AMINO N	PER CENT OF TOTAL N AS FREE NH ₂
	min.	cc.	deg. C.	mm.	mgm.	
Ia	10	1.00	20	762	0.570	1.69
Ib	10	1.00	20	762	0.570	1.69
IIa	30	0.80	25	766	0.448	1.65
IIb	30	0.90	26	758	0.495	1.83

Hemocyanin. The substance was prepared for analysis by grinding to a fine powder, then rubbing it up with 5 per cent sodium carbonate until a colloidal solution was obtained. This was poured into an excess of glacial acetic acid, and the mixture formed a clear solution, which was diluted with water.

Kjeldahl nitrogen: 5-cc. portions; 20.4 cc. $\frac{N}{16}$ HCl. Total nitrogen in 10 cc., 57.12 mgm.

Amino nitrogen: 10 cc. of solution; N gas in thirty minutes, 4.20 cc. at 20°, 765 mm.; amino N, 2.41 mgm.; per cent total nitrogen as amino nitrogen, 4.28.

Zein. The substance was dissolved in glacial acetic acid.

Kjeldahl nitrogen: 10-cc. portions; 24.36 cc. $\frac{N}{16}$ HCl; total N, 34.00 mgm.

Amino nitrogen: 10 cc. of solution gave in thirty minutes the same volume of gas as 10 cc. of glacial acetic acid alone in the control determination. Amino nitrogen not present.

Gliadin. Two grams of gliadin were rubbed up with 5 cc. of glacial acetic acid, the turbid solution diluted to 40 cc. with water, and cleared by centrifugalizing.

Kjeldahl nitrogen: 5-cc. portions; 22.65 cc. $\frac{N}{16}$ HCl, indicating 12.70 mgm. of nitrogen in 2 cc.

Amino nitrogen: 2-cc. portions.

DURATION OF REACTION	N GAS	TEMPERATURE	PRESSURE	AMINO N	PER CENT OF TOTAL N
min.	cc.	deg. C.	mm.	mgm.	
15	0.21	21	764	0.12	0.94
30	0.25	21	764	0.14	1.10

Heteroalbumose (from Witte peptone), dissolved in 0.5 per cent Na₂CO₃ solution.

Kjeldahl determination: 5-cc. portions; 11.65 cc. $\frac{N}{16}$ HCl, indicating 6.52 mgm. N in 2 cc. solution.

Amino nitrogen: 2-cc. portions.

DURATION OF REACTION	N GAS	TEMPERATURE	PRESSURE	AMINO N	PER CENT OF TOTAL N
min.	cc.	deg. C.	mm.	mgm.	
15	0.87	24	762	0.488	7.48
30	0.94	24	762	0.526	8.06

Protoalbumose (from Witte peptone). Solution I. 0.750 gram albumose dissolved in 25 cc. water. Kjeldahl nitrogen: 5 cc. portions; 15.44 cc. $\frac{N}{10}$ HCl, indicating 8.65 mgm. of nitrogen in 2 cc.

Solution II. 0.750 gram albumose dissolved in 25 cc. of 0.5 per cent Na_2CO_3 solution. Kjeldahl nitrogen: 5-cc. portions; 15.49 cc. $\frac{N}{10}$ HCl, indicating 8.67 mgm. of nitrogen in 2 cc.

Amino determinations: 2-cc. portions.

SOLUTION	DURATION OF REACTION	N GAS	TEMPERATURE	PRESSURE	NH ₂ -N	PER CENT OF TOTAL N	CORRECTED FOR NH ₃ IN PREPARATION
	min.	cc.	deg. C.	mm.	mgm.		
I	5	1.34	19	766	0.773	8.94	
II	5	1.32	18	764	0.760	8.77	
I	15	1.70	24	762	0.953	11.03	
II	15	1.53	18	764	0.881	10.16	
I	30	1.74	24	762	0.975	11.28	9.93
II	30	1.67	18	764	0.965	11.13	9.78

The final, thirty-minute results, are no higher when sodium carbonate is used to assist solution (solution II) than when pure water is used (solution I). This fact shows that the dilute sodium carbonate solution had no immediate hydrolyzing effect on the protein.

Unlike the heteroalbumose, the preparation of protoalbumose¹² contained an appreciable amount of free ammonia. Portions of 0.500 gram, used for determination of free ammonia by vacuum distillation from solution made alkaline with calcium hydrate, gave 0.75 and 0.80 cc. of $\frac{N}{10}$ ammonia, equal to 1.46-1.55 per cent of the total nitrogen. In thirty minutes approximately 90 per cent of the nitrogen of ammonia is given off in the amino determination. The free amino nitrogen determined should therefore be reduced by 1.35 per cent of the total to correct for the ammonia.

The figures for free amino nitrogen in hetero- and protoalbumose are higher than those given in the papers from this laboratory on the composi-

¹² Levene, Van Slyke, and Birchard: this *Journal*, viii, p. 269, 1910.

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tion of the albumoses.¹³ The earlier determinations were run for intervals of only five minutes. These suffice for all the other amino-acids, but are not long enough for complete decomposition of the ω -NH₂ group of lysine.

Summary of results.

In the following table the average results of the complete (thirty-minute) determinations are collected. The figures obtained by halving the lysine nitrogen are given for comparison.

PROTEIN ANALYZED	PER CENT OF TOTAL NITROGEN AS	
	Free amino N	One-half the lysine N
<i>A. Native proteins:</i>		
Hemoglobin.....	6.00	5.80*
Casein.....	5.51	5.15†
Hemocyanin.....	4.30	4.25‡
Gelatin.....	3.12	3.15‡
Edestin.....	1.80	1.90‡
Gliadin.....	1.10	0.38‡
Zein.....	0.00	0.00§
<i>B. Albumoses from fibrin:</i>		
Heteroalbumose.....	8.06	5.15**
Protoalbumose.....	• 9.86	4.80**

* Unpublished result.

† Van Slyke: Preceding paper.

‡ Van Slyke: *This Journal*, x, p. 16, 1911.

§ Osborne and Jones: *Ergeb. d. Physiol.*, x, p. 99, 1910.

** Levene, Van Slyke and Birchard: *This Journal*, x, p. 57, 1911.

CONCLUSIONS.

In all the native proteins investigated the amount of free amino nitrogen is equal to one-half the lysine nitrogen, no deviation exceeding the limit of experimental error of the amino and lysine determinations being found in any case with the possible exception of gliadin, in which the difference is 0.7 per cent. The period required for complete reaction of the proteins with nitrous acid (thirty minutes) is longer than that required by the α -amino groups (three to four minutes), but corresponds to that found for

¹³ Levene, Van Slyke, and Birchard: *this Journal*, viii, p. 272, 1910; x, p. 59, 1911.

lysine, with an ω -amino group free. The facts support the following conclusions.

1. One of the two amino groups of lysine, the ω -group, exists free in the protein molecule.

2. This group represents, within at most a fraction of a per cent of the protein nitrogen, the entire amount of free NH_2 determinable in the native proteins by the nitrous acid method.¹⁴ The α -amino groups, which constitute the remaining and greater part of the free amino nitrogen found after complete hydrolysis, are, in the intact protein molecule, practically all condensed into peptide linkings.

3. With the primary albumoses the relations are different. The free NH_2 in hetero- and protoalbumose exceeds half the lysine nitrogen by 3 and 4.8 per cent, respectively, of the total protein nitrogen, indicating that an appreciable portion of the α -amino groups is uncovered in even the primary digestion products.

¹⁴ Osborne, Leavenworth and Brautlecht have demonstrated the probable presence of the acid amide groups of glutamine and asparagine in the protein molecule (*Amer. Journ. of Physiol.*, xxiii, p. 180). Acid amide groups, however, like the guanidine nucleus of arginine, give off none of their nitrogen when treated with nitrous acid, and consequently are not determined by our method.

ON SPHINGOSINE.

SECOND PAPER.

THE OXIDATION OF SPHINGOSINE AND DIHYDROSPHINGOSINE.

BY P. A. LEVENE AND C. J. WEST.

(From the Laboratories of the Rockefeller Institute for Medical Research,
New York.)

(Received for publication, December 1, 1913.)

In 1911 Levene and Jacobs¹ announced the first information regarding the chemical structure of sphingosine. They regarded the substance as a dihydroxy derivative of an unsaturated primary amine. Later Thierfelder and Thomas² corroborated some of these conclusions. Levene and Jacobs³ in their full publication on sphingosine stated that further work on the structure of the base was in progress in this laboratory. It was made clear that the respective positions of the hydroxy groups, of the amino group and of the double bond were under investigation.

The progress of the work was not quite so rapid as expected for the reason that the reduction of dihydrosphingosine into the primary amine offered unexpected difficulties. In the course of these reduction experiments unexpected substances were obtained which may prove of considerable interest and the study of which somewhat delayed the completion of the work.

While this work was in progress there appeared during the course of the present summer a publication by Lapworth⁴ on the structure of sphingosine. The investigations of Lapworth were begun in 1910, but the experiments reported in his publication are apparently based on the knowledge of the structure of the substance furnished by the work of Levene and Jacobs. Lapworth demonstrated that on oxidation of the base with chromium tri-

¹ Levene and Jacobs: this *Journal*, xi, p. xxix, 1912.

² Thierfelder and Thomas: *Zeitschr. f. physiol. Chem.*, lxxvii, p. 511, 1912.

³ Levene and Jacobs: this *Journal*, xi, p. 547, 1912.

⁴ Lapworth: *Journ. Chem. Soc.*, ciii, p. 1029, 1913.

oxide a tridecylic acid was obtained, which he regarded as the normal acid and for this reason argued that in sphingosine the carbon atoms are linked in a straight chain.

Our work on the structure of sphingosine is as yet not completed but we wish to present some of the results of our experiments, particularly in view of the publication of Lapworth. Only those experiments will be discussed in the present communication which deal with the oxidation of sphingosine and dihydrosphingosine. On oxidation of the unsaturated base a tridecylic acid was obtained, while the reduced base under the same conditions of experimentation gave rise to a pentadecylic acid. Regarding the structure of the carbon chains of the two acids, we as yet have no definite information. On the basis of the melting points of the acids one seems justified in concluding that the carbon atoms are not linked in a straight chain, since the normal tridecylic acid has a melting point of 43° ,⁵ the normal pentadecylic acid melts at 53° , while the melting points of our two acids were $47-48^{\circ}$ and $60-61^{\circ}$ respectively. On this point our results do not agree with those of Lapworth. It will be the aim of the future work to establish the exact structure of the carbon chains of these two acids.

Our results, however, are important principally for the reason that through them the position of the double bond is made clear, namely, between the fourth and fifth carbon atoms (from the right end); and further, that through them the possibilities of the position of the two hydroxy and of the amino groups were limited to the carbon atoms 1, 2 and 3. On the ground of this one may express the structure of sphingosine approximately as follows:



The nature of the carbon chain of the part from 5 to 17 is not yet clear and the distribution of the hydroxy and amino groups on the carbon atoms 1-3 is not yet determined. However if the position of a hydroxyl or of an amino group had been removed further than the third carbon atom, then on oxidation of the dihydrobase, instead of the pentadecylic acid, a hydroxy- or an amino-acid should have been formed.

⁵ Le Sueur: *Journ. Chem. Soc.*, lxxxvii, p. 1905, 1905.

EXPERIMENTAL PART.

Our first experiments in the study of the oxidation products of sphingosine were attempts to repeat the work of Lapworth. We added an excess of chromic acid to a glacial acetic acid solution of sphingosine sulphate, keeping the reaction at about 70° on the water bath, the lowest temperature which caused effervescence. The excess of chromic acid was then reduced with sulphur dioxide and the reaction product distilled with steam. Only a very small amount of solid distillate passed over with the steam, 5 grams of sphingosine sulphate giving not more than 0.5 gram of crude acid. There remained in the flask an oily, green residue, soluble in ether, which we attempted to reoxidize, with little success, however. This green product was then boiled with concentrated hydrochloric acid, but it did not give a colorless acid as stated by Lapworth. Since then we have found that this oil contains most of the reaction product which may be obtained by distillation in vacuum, but have not repeated the work under exactly these conditions. In view of this we do not wish to condemn the method until we have tried it again.

After this we tried many conditions of oxidation and finally found the following to be the most suitable. Three grams of sphingosine sulphate were dissolved in about 50 cc. glacial acetic acid, warmed on a boiling water bath to about 85–90°, and a warm solution of 12 grams of chromic acid in 120 cc. glacial acetic acid slowly dropped into it, the flask being shaken quite frequently. After the addition of the acid, the reaction product was diluted with water and distilled with steam until nearly all of the acetic acid had been removed. A small amount of solid acid distilled over with the steam, which, after being dried in ethereal solution, weighed 0.350 gram. The main part of the acid was found in the oily, green residue, which floated on the surface of the dilute acetic acid in the flask. This solidified upon cooling in the ice box, and after drying the ethereal solution weighed 1.03 grams. It contained about 10 per cent ash. The theory from 3 grams sphingosine sulphate is about 1.9 grams.

The green product, when dried in vacuum, gave the following numbers upon analysis, which, calculated on an ash-free substance, indicated the presence of a C₁₃ acid.

0.1170 gram of the substance gave 0.2790 gram CO_2 and 0.1088 gram H_2O . The ash weighed 0.0125 gram.

	Calculated for $\text{C}_{13}\text{H}_{25}\text{O}_2$:	Found:
C.....	72.80	73.17
H.....	12.20	11.82

This dried product was then distilled in vacuum, when a colorless distillate was obtained, which, when distilled twice, was found to be a pure tridecylic acid.

0.1222 gram substance gave 0.3248 gram CO_2 and 0.1338 gram H_2O .

	Calculated for $\text{C}_{13}\text{H}_{25}\text{O}_2$:	Found:
C.....	72.80	72.58
H.....	12.20	12.25

Molecular weight estimation. 0.5300 gram of the acid, dissolved in absolute methyl alcohol and benzene, required 24.8 cc. $\frac{N}{10}$ NaOH for neutralization, using phenolphthalein as an indicator.

	Calculated for $\text{C}_{13}\text{H}_{25}\text{O}_2$:	Found:
Molecular weight.....	214	214

The acid on the last distillation boiled between $190\text{--}200^\circ$, a glass water pump being used. The colorless product melted at $46\text{--}47^\circ$, when cooled and reheated at $46\text{--}47^\circ$ and after standing over night and carefully heated, $47\text{--}47.5^\circ$, the last to be considered as the correct melting point. When recrystallized from dilute acetone the melting point was not changed.

Since the melting point of the acid was higher than that found by Lapworth ($39\text{--}40^\circ$) and differed by $4\text{--}5^\circ$ from the melting point of the normal tridecylic acid (43°), we repeated the oxidation on a larger quantity of sphingosine sulphate. The acid, after the second distillation, melted fairly sharply at $42\text{--}43^\circ$, after recrystallization from pure formic acid at $42\text{--}43^\circ$, but when recrystallized from about 90 per cent acetone, the melting point was raised to $46.5\text{--}47.5^\circ$ as found above.

Oxidation of dihydrosphingosine.

A preliminary experiment was also carried out using dihydrosphingosine. Three grams of dihydrosphingosine sulphate, dissolved in about 125 cc. glacial acetic acid, were treated with 12 grams of chromic acid dissolved in 120 cc. glacial acetic acid and the reac-

tion product worked up as given above. The green mixture was distilled in vacuum and the colorless acid twice redistilled. The product thus obtained melted at 48–49°. When recrystallized from a little dilute acetone it melted at 60–61°. The normal pentadecylic acid, which we prepared from α -hydroxypalmitic acid, melted at 53°. ⁶

0.1288 gram substance gave 0.3506 gram CO₂ and 0.1410 gram H₂O.

0.1459 gram substance gave 0.3154 gram CO₂ and 0.1306 gram H₂O.

0.5282 gram substance required 21.72 cc. $\frac{N}{10}$ NaOH for neutralization.

	Calculated for C ₁₅ H ₃₀ O ₂ :	Found:	
C.....	74.40	74.24	74.28
H.....	12.40	12.25	12.65
Molecular weight.....	242	243.3	

It may be mentioned that Liebermann⁷ describes a pentadecylic acid, melting at 59–60°, which he obtained by the oxidation of cocceryl alcohol. Whether our acid is identical with this acid has not yet been determined.

⁶ Levene and West: this *Journal*, xvi, p. 475, 1914.

⁷ Liebermann and Bergami: *Ber. d. deutsch. chem. Gesellsch.*, xx, p. 959, 1887.



ON THE ACTION OF LEUCOCYTES AND OF KIDNEY TISSUE ON AMINO-ACIDS.

BY P. A. LEVENE AND G. M. MEYER.

(From the Laboratories of the Rockefeller Institute for Medical Research,
New York.)

(Received for publication, December 1, 1913.)

Since the publication of Lang¹ in 1904 it was generally accepted that through the action of tissues amino-acids underwent a deamination which led to the formation of the corresponding hydroxy-acids. It was also considered probable, on the basis of the work of Neubauer,² that the primary product of the reaction is the α -ketonic acid, which is subsequently reduced to the hydroxy-acid. Very recently the work was repeated in Cathcart's laboratory by Gertrude D. Bostock,³ who in the main corroborated Lang's conclusions. This writer, however, makes a passing remark that the liver and intestinal mucosa failed to act on alanine.

Considerations which were discussed in a previous publication⁴ led us to test the action of leucocytes and of various tissues on alanine under conditions in which bacterial growth was completely excluded. It was then definitely proven that under absolutely aseptic conditions, or under conditions of absolutely effective antisepsis, no deamination of alanine took place. Our observation was soon corroborated in Embden's laboratory.⁵ This made it urgent to extend the experiments to a larger number of amino-acids. The experiments were carried out under aseptic conditions, and it was found that not one of the amino-acids tested suffered a deamination through the action of the leucocytes or of the kidney tissue.

¹ Lang: *Hofmeister's Beiträge*, v, p. 321, 1904.

² Neubauer: *Deutsch. Arch. f. klin. Med.*, xcv, p. 211, 1909.

³ Gertrude D. Bostock: *Biochem. Journ.*, vi, p. 48, 1912.

⁴ Levene and Meyer: *this Journal*, xv, p. 475, 1913.

⁵ Griesbach and Oppenheimer: *Biochem. Zeitschr.*, lv. p. 329, 1913.

These observations make it necessary to repeat all the older work on deaminizing action of tissues. Experiments on purine bases are in progress.

EXPERIMENTAL.

Tissues. The leucocytes were obtained from dogs by the injection of turpentine into the pleural cavity. Rabbit kidneys were removed aseptically from exsanguinated animals. The tissues were finely minced before being added to the solutions.

Solutions. Glycocoll, aspartic acid, and leucine in approximately 2 per cent, and asparagine in 1 per cent concentration in 1 per cent Henderson phosphate mixture were used. Two kidneys were used in each experiment and control.

Methods of analysis. The amino-acid nitrogen was determined by the Van Slyke method, total nitrogen by the Kjeldahl process and ammonia nitrogen by distillation *in vacuo*. The details of analysis have been outlined in a previous communication.

Bacteriological control. Aerobic and anaerobic cultures and smears were made of all solutions. We wish to thank Dr. H. L. Amoss for his courtesy.

Kidney.

	ORIGINAL SOLUTION USED	VOLUME GAS	TEMPERA- TURE	PRESSURE	N	N GRAMS PER 100 cc.
	cc.	cc.	deg. C.	mm.	mgm.	
Phosphate solution:						
Before.....	10.0	2.40	22	759	1.35	0.013
After one week.....	10.0	2.60	20	750	1.46	0.015
Glycocoll solution:						
Before.....	2.5	17.10	22	759	9.64	0.385
After one week.....	2.5	17.20	20	750	9.65	0.386
Aspartic acid solution:						
Before.....	5.0	18.00	22	759	10.13	0.203
After one week.....	5.0	18.00	20	750	10.10	0.202
Asparagine solution:						
Before.....	5.0	16.20	20	758	9.30	0.184
After one week.....	5.0	16.20	21	760	9.18	0.184
Leucine solution:						
Before.....	5.0	18.00	20	758	10.24	0.205
After one week.....	5.0	18.30	21	760	10.35	0.207

Leucocytes.

	ORIGINAL SOLUTION USED	VOLUME GAS	TEMPERATURE	PRESSURE	N	N GRAMS PER 100 CC.
Leucocytes and phosphate solution:						
Before.....	2.5	0.20	14	758	0.117	0.0468
After one week.....	2.5	0.20	12	752	0.117	0.0472
Asparagine solution:						
Before.....	2.5	8.50	14	758	4.980	0.1992
After one week.....	2.5	8.40	12	752	4.900	0.1960
Leucine solution:						
Before.....	2.5	11.40	14	758	6.680	0.2672
After one week.....	2.5	11.60	12	752	6.780	0.2712

Kidney.

SOLUTION	AMMONIA N PER CENT			AMINO N PER CENT			TOTAL N PER CENT		
	Before	After	Diff.	Before	After	Diff.	Before	After	Diff.
Phosphate..	0	0.005	0.005	0.013	0.015	0.002	0.025	0.038	0.013
Asparagine	0	0.006	0.006	0.184	0.184	0.000	0.112	0.122	0.010
Glycocoll...	0	0.008	0.008	0.385	0.386	0.001	0.186	0.198	0.012
Leucine.....	0	0.005	0.005	0.205	0.207	0.002	0.175	0.188	0.013
Aspartic acid.....	0	0.005	0.005	0.203	0.202	-0.001	0.366	0.378	0.012

Leucocytes.

Phosphate..	0	0.008	0.008	0.047	0.047	0.000	0.040	0.070	0.030
Asparagine	0	0.012	0.012	0.199	0.196	-0.003	0.168	0.201	0.033
Leucine.....	0	0.010	0.010	0.267	0.271	0.004	0.096	0.131	0.035



ON "SUCRE VIRTUEL" AND BLOOD GLYCOLYSIS.

BY PROF. R. LÉPINE (Lyons),

Correspondent de l'Académie des Sciences de Paris.

(Received for publication, December 5, 1913.)

In an interesting communication which recently appeared in this *Journal*, Macleod¹ states that according to Lépine and Barral "the concentration of actual sugar may be greater in blood that has stood for from fifteen minutes to an hour *at body temperature*,² outside the body than in freshly drawn blood," and he refers to page 64 of my book on diabetes (Paris, 1909). Neither on that page nor elsewhere in any of my publications can be found the words "at body temperature" but exactly in the middle of page 64 there appears the following title: AUGMENTATION DU GLYCOSE DANS LE SANG, *in Vitro*, à 58°. As a matter of fact, it is necessary to inhibit glycolysis and in order to obtain this result a temperature of 58° is essential; *but even this does not always prevent a great loss of sugar in the blood of certain dogs.*³ But leaving aside for the moment exceptional cases, it may be said that if arterial blood be permitted to flow *simultaneously* by means of a bifurcated canula into two tared flasks, one of which, A, contains a known quantity of a solution of mercuric nitrate, while the other, B, immersed in water at 58–59°, contains a weighed quantity of water sufficient to prevent coagulation of the blood; and then if after a quarter, one-half, or even one hour the contents of flask B be poured into a solution of mercuric nitrate and the sugar determined in A and B, it is generally found that the amount of sugar per 1000 grams of blood is greater in B than in A. This

¹ J. J. R. Macleod: this *Journal*, xv, p. 497, 1913.

² Italics are mine.

³ It is not easy to explain this exception. It is certain, as Barral and the writer showed in 1891, that the glycolytic enzyme nearly always loses its activity at 58°. It may be supposed that sometimes the enzyme which decomposes the actual sugar (see below) while continuing its diastatic action decomposes the sugar *which is present in a nascent state*. I advance this hypothesis with great reserve.

slight increase in sugar, commonly observed in healthy and normal dogs (in some instances, as mentioned above, a loss is observed), is much greater in dogs which have previously undergone an operation, particularly in such as have been bled.⁴

The increase in sugar is still greater if a few hours previous to the bleeding the animal is injected subcutaneously or intravenously with a small quantity of pancreatin, invertin, phlorhizin, adrenaline, morphine, or antipyrine—in short with any substance which brings about a rapid modification in the quantity of "sucre virtuel."

More interesting than the increase of sugar *in vitro* is that which occurs in the circulation. I discovered in 1903, in collaboration with Boulud, that in a dog that had been fasting about fifteen hours, the blood of the carotid (*i.e.*, of the left ventricle), contrary to the opinion of Cl. Bernard, very often contains more sugar than the blood of the right ventricle (obtained by means of a sound introduced into the right jugular vein). Many of our numerous experiments have made it possible to state that this increase of sugar in the blood of the carotid takes place at the expense of the combined sugar.⁵ For example:

	SUGAR		
	Free	Combined	Total
Blood of the right ventricle	0.90	0.75	1.65
Blood of the carotid	1.10	0.50	1.60

The increase of sugar in the blood of the renal vein in phlorhizinized dogs discovered by Levene in 1895 (erroneously denied by Zuntz) has the same origin.⁶ Recently we have demonstrated

⁴ Macleod, having kept the blood at body temperature, naturally noted a loss of sugar which was due to normal glycolysis. In any case, if he had greatly increased the number of his experiments he might have found in some exceptional cases a slight gain. I have observed this in two or three instances out of more than one thousand experiments. This can be explained by assuming a considerable decomposition of "sucre virtuel" in an animal whose glycolytic power is weak.

⁵ Regarding the difficulties in estimating the amount of the combined sugar, see Lépine and Boulud: *Journ. d. physiol. et d. path. gén.*, pp. 183-184, 1911.

⁶ Lépine: *Revue d. méd.*, 1913, p. 614, *et seq.*; *Semaine méd.*, Sept. 24; Lépine and Boulud: *Compt. rend. de l'Acad. des Sci.*, October 6, 1913.

that the cleavage of the combined sugar is produced by an enzyme which can be extracted from the vascular wall.⁷ This liberated sugar deserves the name of "sucre virtuel" inasmuch as it is ready to be utilized as soon as it is liberated (apparently spontaneously but in reality under the influence of an enzyme) from the combination in which it was present and in which combination it could not be detected by the ordinary reagents.

The enzyme concerned hydrolyzes phlorhizin. From this fact one is justified in drawing the conclusion that the "sucre virtuel" is of a glucosidic nature.

In reference to glycolysis, Macleod might well have added to his bibliography the article on *glycolysis* in the *Dictionnaire de physiologie* by Richet, and the chapter on *glycolysis* in my book on diabetes (pp. 152-191). According to Macleod the absence of glycolytic power of the serum was discovered by Rona and Döblin. This assertion is inaccurate because I carefully recorded this fact with Barral (in a note at the Académie des Sciences, 1890) and this is furthermore pointed out by Levene and Meyer (this *Journal*, xi, p. 364). With reference to the glycolytic activity of the leucocytes, which I was the first to discover, Van de Put might also be mentioned (*Arch. internat. d. physiol.*, ix, p. 292, 1910). Macleod studied glycolysis *in vitro* after having kept the blood for variable intervals at 40°. In any case, he should have made the estimations after one hour in order to have comparable results. He seems to deny the diminution of glycolysis in the blood of depancreatized dogs. I wish to refer him to page 357 of the *Journal de physiologie et de pathologie générale*, 1911. There he will find an explanation, at least a partial one, of the errors which may be committed in that respect (see also my book, p. 342).

I cannot finish without referring to the important fact discovered by Levene and Meyer that the sugar of the blood can be regained after glycolysis. I have observed an analogous fact (see *Journ. d. physiol. et d. path. gén.*, p. 184, 1911, note by Hugounenq and Morel). But this disturbing factor does not take place in the course of the first hour of glycolysis. Thus, no importance need be attached thereto, if the blood be left for only an hour in the incubator, as has always been my practice.

⁷ Lépine and Boulud: *Compt. rend de l'Acad. des. Sci.*, October 20, 1913.

THE CHEMISTRY OF GLUCONEOGENESIS.

VI. THE EFFECTS OF ACETALDEHYDE AND PROPYLALDEHYDE ON THE SUGAR FORMATION AND ACIDOSIS IN THE DIABETIC ORGANISM.¹

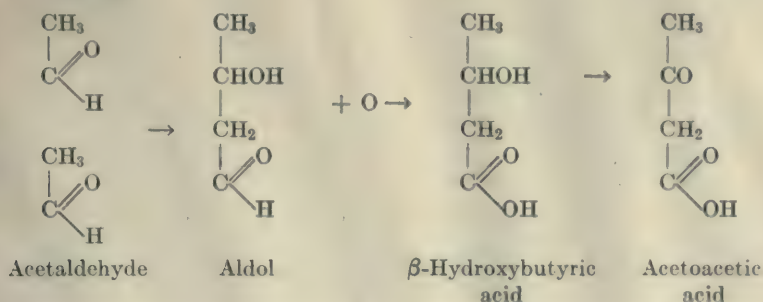
BY A. I. RINGER AND E. M. FRANKEL.

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(Received for publication, December 5, 1913.)

It has long been recognized that aldehydes are capable of effecting a great many syntheses in the animal and plant kingdoms. Thus, formaldehyde is generally accepted now to be the building stone from which sugars are synthesized in the plant kingdom² and Grube³ demonstrated the possibility of this synthesis taking place in the liver of the turtle perfused with a fluid containing formaldehyde.

The first suggestion that acetaldehyde may play a rôle in the synthetic processes of the animal body was made by Spiro.⁴ He suggested the possibility of β -hydroxybutyric acid arising from a condensation of two molecules of acetaldehyde, going through an aldol stage.



¹ Aided by a grant from the Rockefeller Institute for Medical Research.

² v. Bayer: *Ber. d. deutsch. chem. Gesellsch.*, iii, p. 63, 1870.

³ Grube: *Pflüger's Archiv*, cxxi, p. 636, 1908; cxxxix, p. 428, 1911.

⁴ Spiro, quoted by Magnus-Levy: *Arch. f. exp. Path. u. Pharm.*, xlii, p. 225, 1899.

Friedman⁵ subjected these views to the test of experimentation and found that the perfusion of a dog's liver with blood to which acetaldehyde had been added, was actually followed by an increase in the acetoacetic acid in the perfusion mixture. On testing the effects of aldol he likewise obtained a very marked increase in the acetoacetic acid.

We were led to the study of the fate of acetaldehyde in the diabetic dog while searching for possible intermediary compounds in the metabolism of pyruvic acid. As was shown in a previous communication⁶ pyruvic acid in different experiments does not yield glucose to the same degree. Several paths of catabolism of pyruvic acid suggested themselves, and we hoped to be able to show that pyruvic acid passes in part through acetaldehyde when it gives rise to only small quantities of glucose. The experiments, however, yielded entirely unexpected and contrary results.

The methods used in these experiments were the same as described in the previous communications of this series. Female dogs were used which were phlorhizinized by daily injection of 1 gram of phlorhizin ground up in olive oil. The animals were catheterized at the end of each period of twelve hours, after which the bladder was washed three or four times with warm distilled water. The acetaldehyde used was prepared by Merek (derivative of absolute alcohol) and was given subcutaneously diluted with water. In several instances the acetaldehyde was redistilled immediately before injection.⁷ The nitrogen was determined by Kjeldahl, glucose by Alihn, ammonia by Folin, acetone, acetoacetic acid and β -hydroxybutyric acid by Shaffer's methods. The glucose determination was controlled by the polariscopic method, and after aldehyde feedings was also controlled for its fermentability by yeast.

Effect of acetaldehyde, CH_3-CHO .

In experiment XXVII, period XIV, 8.8 grams ($-\frac{M}{5}$) of acetaldehyde dissolved in 35 cc. of water were given subcutaneously. From

⁵ Friedman: *Hofmeister's Beiträge*, xi, p. 202, 1908.

⁶ Ringer: *This Journal*, xv, p. 145, 1913.

⁷ Attention must be called to the fact that acetaldehyde undergoes considerable deterioration on standing, which lessens its effects very materially.

the results of this experiment we see that the acetaldehyde exerts a very profound influence on the nitrogen as well as the glucose elimination. The nitrogen excretion of this dog which stayed above 5 grams per period for thirteen periods was reduced to 3.02 and 3.52 grams in periods XIV and XV to rise again to 6.60 grams in period XVI. The glucose elimination which was 18.75 grams in the foreperiod rose in the experimental period to 21.07 grams in spite of the very marked drop in the nitrogen excretion. The D : N ratio rose from its level of 3.65 in the foreperiod to 7.19 and 4.62 to come down again to 3.03 in period XVI. *The amount of extra glucose eliminated in periods XIV and XV was 16.10 grams.*

In experiment XXVIII, period IX, 8.8 grams ($\frac{8}{5}$) of acetaldehyde dissolved in 25 cc. of water were given subcutaneously. Here as in the preceding experiment we note a depression in the nitrogen and a rise in the glucose elimination. The nitrogen elimination which was 6.42 grams in the foreperiod (period VIII) was reduced to 5.03 and 4.95 grams in period IX and X respectively to come up again to 6.18 and 6.23 in periods XI and XII. The glucose elimination which was 22.39 grams in the foreperiod, rose to 29.70 in spite of the reduction in the nitrogen elimination. The D : N ratio rose from 3.48 to 5.91 and 4.89 to come down again in periods XI and XII to 3.54 and 3.40. *The amount of extra glucose eliminated in periods IX and X was 18.9 grams.*

In this experiment the effect of acetaldehyde on the acidosis was also studied. As is seen from the table, page 576, acetaldehyde possesses a very marked antiketogenetic effect. The amount of β -hydroxybutyric acid elimination in the foreperiod was 2.35 grams. After the administration of acetaldehyde it was reduced to 1.42 and 0.50 gram in periods IX and X respectively, to come up again to 1.50 and 1.72 in periods XI and XII. Similar was the effect on the acetone and acetoacetic acid elimination. In period VIII (foreperiod) 630 mgm. of the two ketones were eliminated. After the acetaldehyde administration in periods IX and X it was reduced to 360 and 180 mgm. to rise again in periods XI and XII to 380 and 470 mgm. There was also a reduction in the ammonia elimination following the acetaldehyde administration. This was relative as well as absolute.

In experiment XXIX, period III, 8.8 grams ($\frac{8}{5}$) of acetaldehyde dissolved in 28 cc. of water were given subcutaneously.

The results of this experiment corroborate our findings in the preceding two experiments. The nitrogen elimination which was 5.15 grams in the foreperiod (period II) was reduced to 3.27 and 2.77 in periods III and IV and rose again to 6.16 and 6.53 grams in periods V and VI. The glucose elimination, in spite of a very marked drop in the nitrogen output, rose from 17.59 in the foreperiod to 24.85 grams in period III resulting in a D : N ratio of 7.61. *The amount of extra glucose eliminated in periods III and IV was 20.45 grams.* The effect of the acetaldehyde on acidosis was also studied in this case. Although distinct, it was, however, not as marked as in experiment XXVIII. The reason for this probably lies in the fact that the elimination of acetone bodies in this dog was very low to start with.

In experiment XXX, period XII, 5.0 grams of acetaldehyde dissolved in 20 cc. of water were given subcutaneously. Here too the elimination of the acetone bodies and of nitrogen was depressed very considerably. The rise in the glucose output, however, was very slight, resulting in only 3.2 grams of extra glucose. It is noteworthy that this dog had a very low (for phlorhizin glucosuria), almost abnormal D : N ratio to start with, and we are inclined to believe that the failure to yield more extra glucose may have some relationship to it.

In experiment XXXI, period II, 8.8 grams ($-\frac{M}{5}$) of acetaldehyde dissolved in 30 cc. of water were given subcutaneously. There followed only a slight diminution in the nitrogen elimination, but a considerable rise in the glucose output. *The amount of extra glucose eliminated in periods II and III was 10.7 grams.*

On examining the tabulated results of these experiments we note a number of very striking effects brought about by the acetaldehyde.

I. A very marked depression of the nitrogen elimination which lasts for about two periods (twenty-four hours) after the acetaldehyde administration.

II. A rise in the absolute amount of glucose eliminated during the period of acetaldehyde administration, in spite of the drop in the nitrogen, accompanied by a very high rise in the D : N ratio.

III. A very marked depression in the acetone, acetoacetic acid and β -hydroxybutyric acid eliminations where acidosis is high.

As will be seen from a subsequent communication the admin-

istration to diabetic dogs of ethyl alcohol and acetic acid which stand in such very close chemical relationship to acetaldehyde are not followed by any of the effects enumerated above. This suggested the possibility that the aldehyde radical $-\text{C} \begin{array}{l} \text{O} \\ \diagup \\ \text{H} \end{array}$ may be responsible for the effects brought about by the acetaldehyde. We, therefore, decided to study the effects of its homologues.

Effect of propylaldehyde, $\text{CH}_3-\text{CH}_2-\text{CHO}$.

In experiment XXVII, period XVII, 11.6 grams ($\frac{M}{3}$) of Kahlbaum's propylaldehyde dissolved in 30 cc. of water to which 3 cc. of ethyl alcohol had been added, were given subcutaneously. The results were very similar to those obtained with acetaldehyde. There followed a very marked depression in the nitrogen elimination and a rise in the D:N ratio. *The amount of extra glucose eliminated in periods XVII and XVIII was 11.65 grams.*

Much more convincing results were obtained in experiment XXVIII. In period XIII of this experiment 11.6 grams ($\frac{M}{3}$) of propylaldehyde were administered as above. The nitrogen elimination which stood at the level of 6.18 and 6.23 in periods XI and XII dropped down to 3.27 and 4.83, to rise again to 6.24 grams in period XV. The glucose elimination in period XIII rose to 24.02 grams in spite of the very marked reduction in the nitrogen elimination, resulting in a rise in the D:N ratio to 7.35. *The amount of extra glucose eliminated was 19.09 grams.*

The effect of propylaldehyde on the ammonia, acetone, acetoacetic acid and β -hydroxybutyric acid eliminations was as marked as that of the acetaldehyde. All were depressed very considerably. The ammonia nitrogen elimination which was 0.65 and 0.70 gram in periods XI and XII was reduced to 0.25 gram in period XIII. The reduction of the ammonia nitrogen from 10.5 and 11.2 per cent to 7.6 per cent of the total nitrogen is also noteworthy. The acetone and acetoacetic acid elimination was reduced from 380 mgm. in period XI and 470 mgm. in period XII to 120 mgm. in periods XIII and XIV. The β -hydroxybutyric acid elimination which was 1.50 and 1.72 grams in the two foreperiods was reduced to 0.36 and 0.25 gram in periods XIII and XIV.

In experiment XXXII, period VI, 11.6 grams of propylaldehyde were administered as above. The results are very similar to those obtained in the preceding experiment. The glucose elimination and the D:N ratio rose very considerably, *yielding 19.75 grams of extra glucose*. The reduction in the acetone bodies was very marked. The acetone and acetoacetic acid having come down from 640 mgm. to a little above 200 mgm. while the β -hydroxybutyric acid was reduced from 1.87 grams in period V to 0.98 gram and 0.378 gram in periods VI and VII respectively.

Effect of formaldehyde, H-CHO.

We have attempted several experiments to study the effect of formaldehyde on the diabetic dog, but all have failed so far because of the toxicity of the aldehyde. The animal usually dies within twelve hours. We hope, however, to come back to these experiments in the near future by administering the aldehyde in very small quantities at a time. Perhaps this may yield satisfactory results.

Discussion of results.

From a review of the preceding pages we note a very remarkable reaction which apparently seems to be characteristic of substances possessing an aldehyde radical, for neither the alcohols, nor the acids corresponding to the aldehydes studied possess the power of effecting such deep seated changes in the metabolism of the diabetic animal. It is true that propyl alcohol and propionic acid possess the power of glucogenesis but they do not affect the nitrogen metabolism nor the acidosis, to the extent that propylaldehyde does. The effect of the acetaldehyde is much more remarkable, because neither the alcohol nor the acid that corresponds to it has any appreciable influence on the metabolism of the diabetic dog.

The outcome of our experiments leads to conclusions diametrically opposed to those of Friedman.⁸ As was stated above he found that acetaldehyde on perfusion through the surviving liver gives rise to acetoacetic acid. On giving acetaldehyde subcutaneously, however, we found that it has just the opposite effect. How can we explain these differences?

⁸ Friedman, *loc. cit.*

It is possible that in perfusion of the surviving extirpated liver one may deal with a metabolism that is decidedly abnormal, and which does not correspond with the process in the same organ when in normal condition. Under such circumstances, we can readily understand, why a substance should follow one path of metabolism in one case and an entirely different one in the other. It must also be borne in mind in this connection that the functions of the liver in the animal body may constantly be influenced by the other organs or by products of their metabolism. An illustration of such influence can be found in the works of Levene and Meyer⁹ who showed how essential the coöperation of the different organs is for carbohydrate metabolism, and also to what erroneous and misleading conclusions one may be drawn by studying the influences of individual organs on the processes of metabolism, without taking into consideration the possible influence of the other organs upon the one studied.

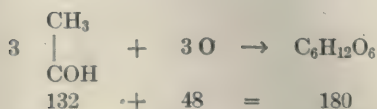
But most of the results obtained by Embden and his collaborators on perfused surviving livers have been found in a general way to be identical with those obtained in feeding experiments. Is it not possible, therefore, that in Friedman's experiments another factor played a rôle which is of great moment in determining the path of the metabolism of acetaldehyde? Is it not possible, for example, that the presence or absence of glycogen in the liver may have influenced his results? Surely, this is a matter which requires further study, especially since it was shown by Friedman¹⁰ that the perfusion of sodium acetate through a liver poor in glycogen will cause an increase in the acetoacetic acid formation whereas the perfusion through a liver rich in glycogen will be followed by negative results.

The glucogenetic effect of acetaldehyde and propylaldehyde.

One of the most remarkable phenomena in our experiments is the very large amount of "extra" glucose that was eliminated after the administration of the acetaldehyde. If all the carbon of the acetaldehyde molecule were converted into glucose the administered 8.8 grams would give rise to 12 grams of glucose.

⁹ Levene and Meyer: *This Journal*, ix, p. 97; xi, pp. 347, 353, 361; xii, p. 265.

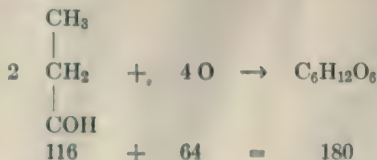
¹⁰ Friedman: *Biochem. Zeitschr.*, lv, p. 436, 1913.



The amount of "extra" glucose found in experiment XXVII was 16.10 grams, in experiment XXVIII, 18.9 grams, in experiment XXIX, 20.45 grams and in experiment XXXI, 10.7 grams.

From this we see very clearly that considerably more glucose was eliminated in the urine after acetaldehyde administration, than can be accounted for by a complete conversion of the acetaldehyde into glucose. In other words, some substance or substances that are ordinarily non-gluco-genetic have contributed to the formation of glucose. The conclusion, therefore, seems justified that *acetaldehyde possesses the power of converting some substance in the animal metabolism that is non-gluco-genetic to one that is gluco-genetic and that the substance so formed possesses a greater number of carbon atoms than does acetaldehyde.* Whether acetaldehyde itself takes part in the gluco-genetic process will be discussed later.

What is true for acetaldehyde is also true for propylaldehyde, but to a lesser extent. If all the carbon of the propylaldehyde were converted into glucose 11.6 grams of propylaldehyde could yield 18 grams of glucose.



The amount of "extra" glucose obtained in experiment XXVII was 11.65 grams, in experiment XXVIII, 19.10 grams and in experiment XXXII, 19.75 grams. The "extra" glucose in experiment XXVII is lower than in any of the others and may be accounted for by the fact that it was performed on a dog that had had glucosuria for seventeen experimental periods, outside of the preparatory periods. At this time the animals find themselves in a very low state of vitality and this may account for the difference. The results obtained at this stage are usually taken for corroborative purposes only.

In experiments XXVIII and XXXII there is clearly a greater amount of glucose eliminated than can be accounted for by a complete conversion of the propylaldehyde into glucose, and similar to the acetaldehyde it seems to possess the power of converting non-glucogenetic substances into glucogenetic ones.

The question arises now, what is the nature of this change? In what way may the acetaldehyde or propylaldehyde exert its influence upon non-glucogenetic substances? Does the acetaldehyde or propylaldehyde bring about its effects by modifying the normal path of metabolism of those substances, or does it enter with them into a chemical union, thus changing their structural configuration and thereby modifying their path of catabolism?

When we come to examine the effects of the aldehydes on the diabetic organism as a whole and associate the different phenomena, a theory suggests itself which seems to harmonize all the facts. Our experiments bring to light three important facts:

I. That the administration of aldehydes in diabetic animals is followed by a very marked rise in the glucose elimination.

II. That concomitant with this phenomenon there is a considerable drop in the elimination of acetone bodies.

III. That alcohols and acids related to the aldehydes do not possess these effects.

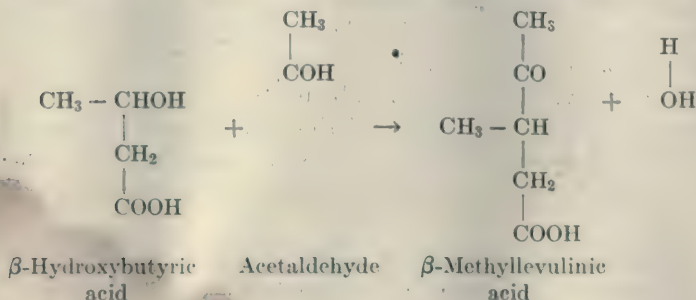
From the above it becomes evident that the aldehyde radical

$\text{—C} \begin{array}{l} \nearrow \text{O} \\ \searrow \text{H} \end{array}$ is the determining factor in bringing about the effects

described. In a subsequent communication additional evidence will be presented in support of this view. The aldehyde radicals possess great combining powers and it is generally recognized now, in what a complexity and multiplicity of unions the aldehydes are capable of entering. In the disaccharides, glucosides, and glucuronates and a great many other compounds this fact is evident.

When we bear this fact in mind and realize that the acetaldehyde and propylaldehyde in our diabetic animals have brought about a diminution in the acetone bodies on the one hand and an increase of the glucose elimination on the other, it seems reasonable to assume that the two phenomena may be causally related,

i.e., the aldehydes, because of their great combining power, may have the property of combining with the secondary alcohol radical of β -hydroxybutyric acid, and by changing its structural configuration convert it into a substance that is glucogenetic. An illustration of the possibility is given in the following reaction,



which results in the formation of β -methyllevulinic acid, *i.e.*, the conversion of a normal fatty acid into an iso compound. It has been shown by Baer and Blum, Embden and his collaborators, and by Ringer, Frankel and Jonas¹¹ that the iso compounds in the animal body undergo demethylation. β -Methyllevulinic acid would therefore be converted into levulinic acid, which, as will be shown in a subsequent communication, does possess glucogenetic properties.

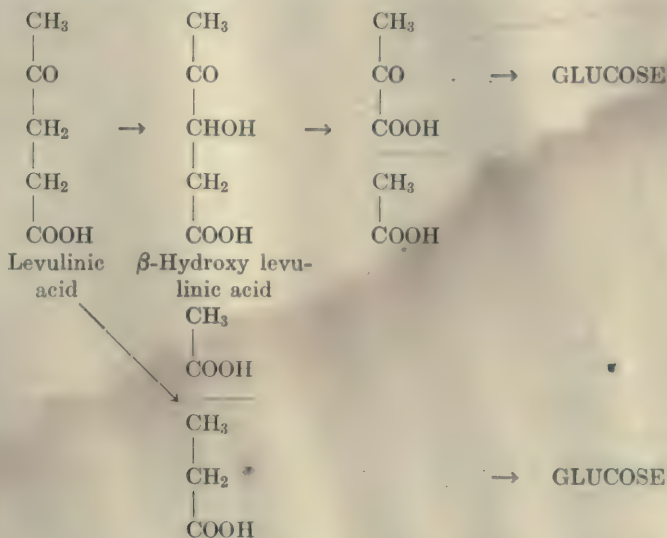
It must be realized that between the β -hydroxybutyric acid and acetaldehyde combination and levulinic acid there may be a number of possible intermediary compounds of the keto and enol forms which will be discussed elsewhere. At present we wish only to sketch our conception of the possible reaction and indicate the possibility of the conversion of a compound with an even number of carbon atoms as β -hydroxybutyric acid to one with an uneven number of carbon atoms, the conversion of an acetone-genetic compound to one that is glucogenetic.

Objection may be raised to this theory because the increase of glucose elimination in our experiment is much greater than is the

¹¹ Baer and Blum: *Arch. f. exp. Path. u. Pharm.*, lv, p. 89, 1906; Embden, Salomon and Schmidt: *Hofmeister's Beiträge*, viii, p. 129; Ringer, Frankel and Jonas: *This Journal*, xiv, p. 525, 1913.

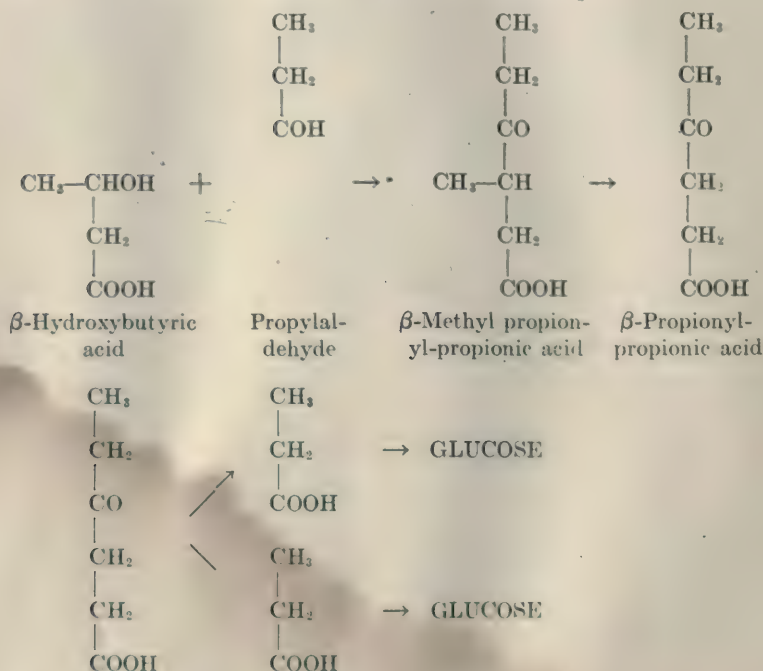
drop in the acetone elimination. But as will be shown in a subsequent communication, this objection is not valid. For, the acetone bodies that are eliminated in the urine are only a small fraction of that which actually plays a part in the intermediary metabolism, and any change that is evident in the urine may be greatly magnified in the intermediary metabolism.

The path of levulinic acid in metabolism is the subject of our present inquiry. It may be one of the following; it may undergo β -oxidation giving rise to pyruvic acid, or it may undergo further



oxidation in the γ -carbon, breaking up into acetic acid and propionic acid. In either case we get a three-carbon compound which is glucogenic. Judging from the amount of glucose we obtain after acetaldehyde administration we are inclined to believe that propionic acid is the final product.

In the case of the propylaldehyde we may conceive of the following reactions:



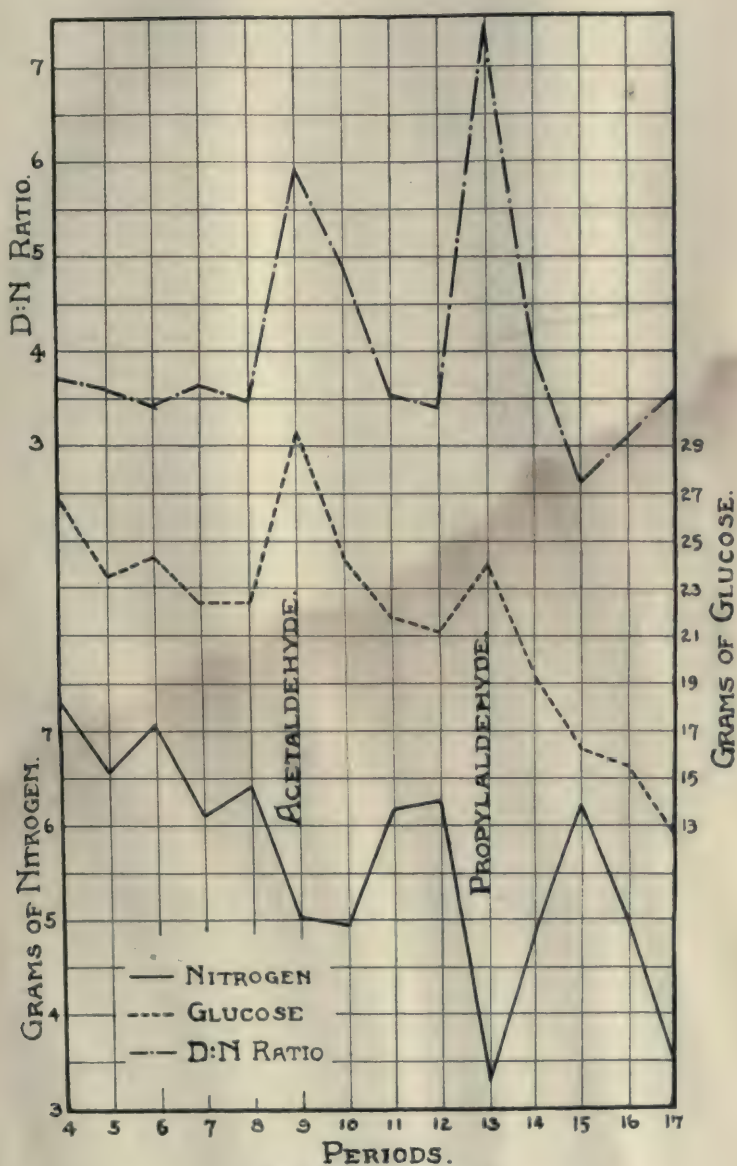
These experiments have opened up a new field for experimentation which, in our estimation, throws a great deal of light on the mechanism of antiketogenesis. They also bring up the question of the influence of the higher aldehydes on metabolism. Most of these experiments are completed and will be published in the near future. The effect of the aldehydes on the protein metabolism will be discussed in a separate communication.

EXPERIMENT XXVII. *Twelve-hour periods.*

DATE MAY, 1913	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	D: N	EXTRA GLUCOSE	REMARKS
18	VIII		6.15	24.26	3.95		
18	IX	11.35	6.42	23.95	3.73		
19	X		6.15	21.30	3.46		
19	XI	10.90	5.82				
20	XII		5.26	17.55	3.34		
20	XIII	10.64	5.14	18.75	3.65		
21	XIV		3.02	21.70	7.19	16.10	8.8 grams acetaldehyde dissolved in 30 cc. of water given subcutaneously.
21	XV		3.52	16.24	4.62		
22	XVI		6.60	19.87	3.03		
22	XVII		3.14	15.64	4.98	11.65	11.6 grams propylaldehyde dissolved in 30 cc. of water to which 3 cc. of ethyl alcohol had been added, given subcutaneously.
23	XVIII		3.78	15.04	3.98		
23	XIX		3.12	7.74	2.48		

EXPERIMENT XXVIII. *Twelve-hour periods.*

DATE MAY, 1913	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	D: N	EXTRA GLUCOSE	AMMONIA NITROGEN	NH ₃ -N: TOTAL N IN %	ACETONE AND ACETO- ACETIC ACID	β -HYDROXY BUTYRIC ACID	REMARKS
18	IV	12.13	7.33	26.96	3.68		0.49	6.7	0.30	1.75	
19	V		6.56	23.50	3.58		0.44	6.7	0.32	1.86	
19	VI	11.48	7.07	24.38	3.45		0.40	5.7	0.40		
20	VII		6.11	22.36	3.66		0.61	10.0	0.76		
20	VIII	11.00	6.42	22.39	3.48		0.73	11.3	0.63	2.35	
21	IX		5.03	29.70	5.91		0.43	8.5	0.36	1.42	8.8 grams acet- aldehyde dis- solved in 25 cc. of water given subcutaneous- ly.
21	X	10.53	4.95	24.20	4.89	18.9	0.41	8.3	0.18	0.50	
22	XI		6.18	21.88	3.54		0.65	10.5	0.38	1.50	
22	XII	10.45	6.23	21.14	3.40		0.70	11.2	0.47	1.72	
23	XIII		3.27	24.02	7.35		0.25	7.6	0.12	0.36	11.6 grams of pro- pyl aldehyde dissolved in water and alco- hol as above, given subcu- taneously.
23	XIV		4.83	19.45	4.03	19.09			0.12	0.25	
24	XV		6.24	16.27	2.62				0.15	0.35	
24	XVI	9.77	4.99	15.50	3.11				0.23	0.51	
25	XVII		3.55	12.76	3.60						



EXPERIMENT XXVIII.

EXPERIMENT XXIX. Twelve-hour periods.

DATE JUNE, 1913	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	D. N	EXTRA GLUCOSE	ACETONE AND ACETO- ACETIC ACID	β -HYDROXY BUTYRIC ACID	REMARKS
3	I								
3	II	11.00	5.15	17.59	3.42		0.070	0.143	
4	III		3.27	24.85	7.61	20.45	0.078	0.200	
4	IV		2.77	13.90	5.02		0.062	0.170	
5	V		6.16	16.24	2.64		0.043	0.143	8.8 grams acetaldehyde dissolved in 28 cc. of water given subcuta- neously.
5	VI	10.68	6.53	18.43	2.82		0.038	0.090	
							0.079	0.184	

EXPERIMENT XXX. Twelve-hour periods.

DATE MAY, 1913	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	D. N	EXTRA GLUCOSE	ACETONE AND ACETO- ACETIC ACID	β -HYDROXY BUTYRIC ACID	REMARKS
9	VII								
10	VIII	8.55	3.59	8.98	2.50		0.33	0.79	
10	IX	8.36	3.53	8.56	2.44		0.47	1.34	
11	X		2.62	7.20	2.75				
11	XI	8.34	2.79	7.60	2.72		0.36	1.13	
12	XII		1.97	7.83	3.97	3.2	0.14	0.38	5.0 grams acetaldehyde dissolved in 20 cc. of water given subcu- taneously.
12	XIII		1.77	5.57	3.14		0.06	0.11	

EXPERIMENT XXXI. Twelve-hour periods.

DATE MAY, 1913	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	D: N	EXTRA GLUCOSE	ACETONE AND ACETO- ACETIC ACID	β -HYDROXY BUTYRIC ACID	REMARKS
14	I		5.97	22.50	3.77		0.06	0.18	8.8 grams acetaldehyde dissolved in 30 cc. of water given subcu- taneously.
15	II		5.49	26.35	4.80	10.7	0.10	0.39	
15	III	12.76	5.76	26.26	4.56		0.07	0.18	
16	IV		7.80	28.60	3.68		0.13	0.35	

EXPERIMENT XXXII. Twelve-hour periods.

DATE OCTOBER, 1913	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	D: N	EXTRA GLUCOSE	ACETONE AND ACETO- ACETIC ACID	β -HYDROXY BUTYRIC ACID	POLARI- ZATION	REMARKS
17	IV	15.24	7.25	28.65	3.96		0.735	2.73	+1.06°	11.6 grams propylaldehyde dissolved in water and alcohol as above given subcutaneously.
17	V		7.90	29.37	3.72		0.640	1.87	+1.19°	
18	VI	14.96	7.04	38.26	5.44	19.75	0.288	0.98	+1.53°	
18	VII		6.32	32.40	5.12		0.211	0.38	+1.33°	
19	VIII	15.02	8.70	33.96	3.90		0.216	0.38	+1.42°	
19	IX		8.75	29.19	3.34		0.202	0.31	+1.24°	



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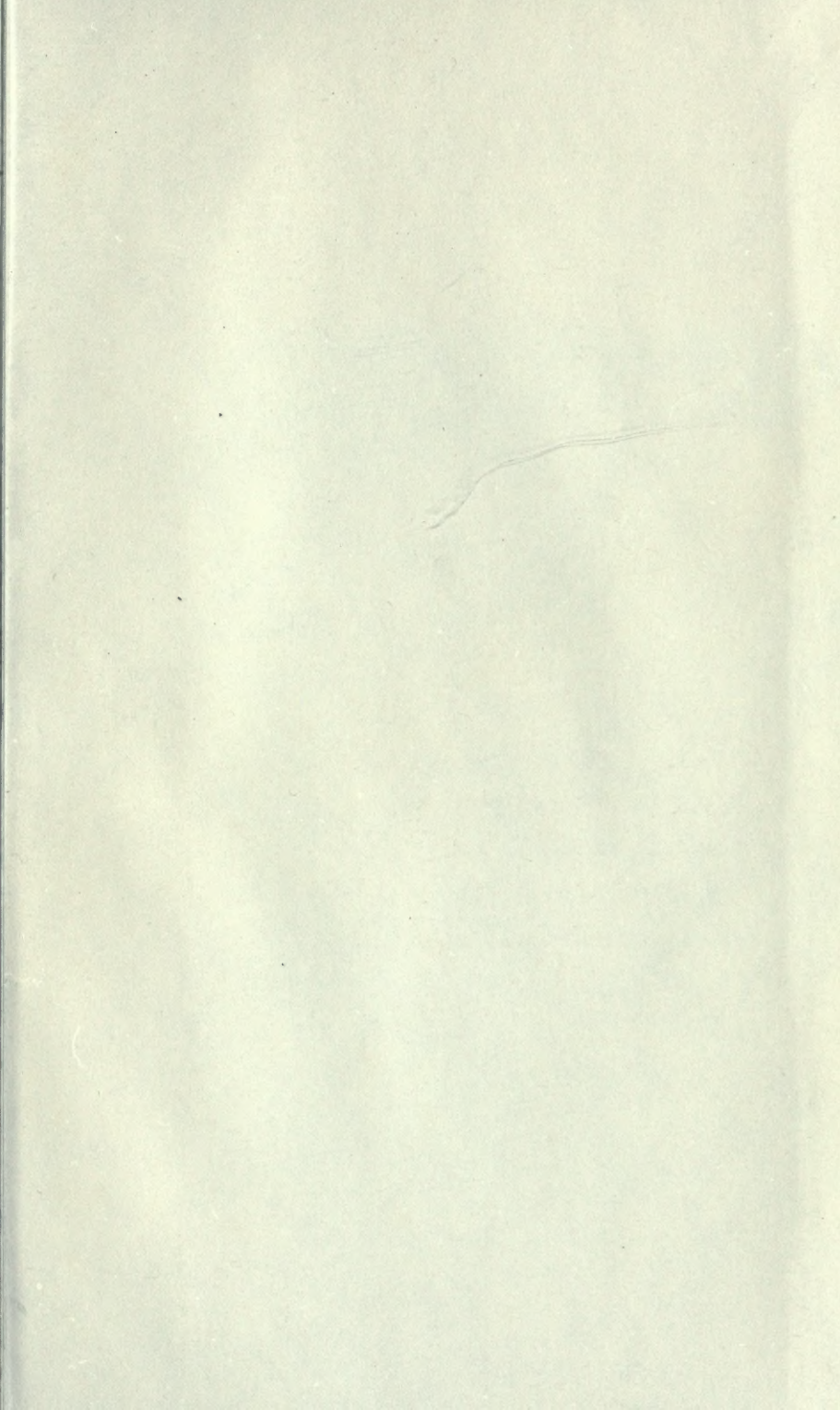
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